The glucose transporter of the bacterial phosphotransferase system couples vectorial translocation to phosphorylation of the transported sugar. It consists of a transmembrane subunit (IICB$^{\text{Glc}}$) and a hydrophilic subunit (IIA$^{\text{Glc}}$). The IICB$^{\text{Glc}}$ subunit consists of two domains. The NH$_2$-terminal IIC domain (residues 1-386) spans the membrane eight times and contains the substrate binding site. The COOH-terminal hydrophilic IIB domain (residues 391-476) is accessible from the cytoplasmic side of the membrane. It contains the phosphorylation site (CYS$^{342}$) and together with the IIC domain catalyzes the transfer of phosphoryl groups from the IIA$^{\text{Glc}}$ subunit to the transported solute. Starting from a plasmid vector containing pStG under an inducible promoter, the IIB and the IIC domains have been subcloned separately, overexpressed, and purified by Ni$^{2+}$ chelate affinity chromatography. Approximately 40 mg of IIB$^{\text{Glc}}$-6H and 4 mg of IIC$^{\text{Glc}}$-6H could be purified from 1 liter of culture. Cells expressing IIB$^{\text{Glc}}$-6H and IIC$^{\text{Glc}}$-6H separately have a three times longer generation time on glucose minimal medium than cells expressing wild-type IICB$^{\text{Glc}}$. The rate of IIB$^{\text{Glc}}$-6H phosphorylation determined in a nitrocellulose filter binding assay is indistinguishable from wild-type IICB$^{\text{Glc}}$. The in vitro specific activity of IIC$^{\text{Glc}}$-6H in the presence of excess IIB$^{\text{Glc}}$-6H is 2% of the control. IIB$^{\text{Glc}}$-6H also complements the activity of a IICB$^{\text{Glc}}$ mutant with an inactive IIB domain (C421S) indicating that IIC and IIB are flexibly linked such that a free IIB domain can displace an inactive IIB domain from its contact site on the IIC domain. Based on this work, the secondary structure of the IICB$^{\text{Glc}}$ domain is highly conserved across domains and functional units. The three-dimensional structures of the soluble IIA$^{\text{Glc}}$ subunit from E. coli and Bacillus subtilis have already been determined by x-ray and NMR methods (Pelton et al., 1991a, 1991b, 1992; Worthylake et al., 1991; Fairbrother et al., 1991a, 1991b, 1991c, 1991d, 1991e). A recombinant hybrid protein (IIA$^{\text{Glc}}$-IIB$^{\text{Glc}}$NAc) which comprises the amino-terminal two-thirds of IICB$^{\text{Glc}}$ subunit and the complementary carboxyl-terminal half of IICB$^{\text{Glc}}$NAc was found to be glucose-specific. The fusion junction was in a highly conserved sequence, LKT-PGRED, which was proposed to act as interdomain linker (Hummel et al., 1992).

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The glucose transporter of Escherichia coli acts by a mechanism that couples translocation with phosphorylation of the substrate. It belongs to the family of transporters known as enzymes II of the bacterial phosphotransferase system (for comprehensive reviews, see Postma (1987), Meadow et al. (1990), and Postma et al. (1992)). These transporters consist of three functional units (IIA, IIB, IIC; Saier and Reizer (1992)) which are either subunits of a complex or domains of a multicomponent polypeptide. The hydrophobic unit (IIC) spans the membrane and contains the sugar binding site. The two hydrophilic units (IIA, IIB) are on the cytoplasmic face of the inner membrane and contain one phosphorylation site each. The transporters differ in amino acid sequence, substrate selectivity, the chemical nature of the IIB phosphorylation site, and in the organization of the functional units as domains and subunits (reviewed by Robillard and Lolkema (1988), Erni (1992), and Postma et al. (1992)). The transporters are continuously phosphorylated by two cytoplasmic proteins, enzyme I and HPr, which in this order sequentially transfer phosphoryl groups from phosphoenolpyruvate to phosphoenolpyruvate. Histidines are the phosphorylation sites on enzyme I, HPr, and the IIA domains of the different transporters (Weigel et al., 1982a, 1982b; Dörschug et al., 1984). A histidine or a cysteine is phosphorylated at the active site of the IIB domain depending on the particular transporter (Pas and Robillard, 1988; Erni et al., 1989, Pas et al., 1991; Meins et al., 1993).

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The Domains of $\text{IIC}^{\text{Gle}}$

**Fig. 1. Hypothetical model of $\text{IIC}^{\text{Gle}}$.** The membrane topology is based on PhoA and LacZ fusions (Buhr and Erni, 1993). The secondary structure of the cytoplasmic IIB domain has been determined by NMR methods (Golic Grdadolnik et al., 1994). Cys421 is the phosphorylated residue from which the phosphoryl group is transferred to the transported sugar. The proposed linker sequence LKTPGRED between the IIC and IIB domain is indicated by the asterisk and the position of the cleavage is indicated by the arrow.

crystallised and is too large for NMR analysis. A topological model of its transmembrane domain (Fig. 1) has been derived from protein fusion experiments (Buhr and Erni, 1993). In order to facilitate the structural analysis in particular of the IIB domain, the $\text{IIC}^{\text{Gle}}$ subunit was dissected into two domains. Both could be overexpressed and purified in active form. Based on this work the secondary structure of the IIB domain has been determined by heteronuclear NMR spectroscopy (Golic Grdadolnik et al., 1994). The IIB domain consists of four $\beta$-strands forming an antiparallel $\beta$-sheet, two large $\alpha$-helices at the NH$_2$ and COOH termini and a smaller helical structure between $\beta$-sheet II and III (Fig. 1). Structural refinements and further NMR studies are in progress.

**EXPERIMENTAL PROCEDURES**

Bacterial Strains and Growth Media—In order to prevent recombination between the mutated chromosomal ptsG allele of $\text{ZSC112L}$ ptsG $\text{glk manZ}$, Curtis and Epstein (1975) and plasmid-encoded ptsG variants, chromosomal ptsG was deleted by site-specific recombination with a linear DNA fragment encoding cat flanked by up- and downstream sequences of ptsG. Gene replacement was achieved as follows. (i) A DNA fragment encoding cat was isolated with BssHI and Sall from plasmid pMe 5–8 (Stanisens et al., 1989), blunted, and inserted into the HpaI site 0.5 kilobases downstream of the ptsG coding region of plasmid pTSG10 (Erni and Zanolori, 1986). (ii) From this new plasmid the ptsG coding region was restricted with NaiI and SmaI followed by self-ligation affording a plasmid with cat now flanked by a total of 4 kilobases chromosomal DNA. (iii) The plasmid was digested with AuckI and the DNA fragment containing cat and flanking chromosomal DNA was used to transform DPE271 (recD; Russel et al., 1989). Transformants defective in glucose uptake (DPE271AG) were selected on McConkey agar plates containing chloramphenicol and glucose. Replacement of ptsG by cat resulted in pale red color of the colonies. (iv) The cat gene was transferred by P1 transduction from DPE271AG into $\text{ZSC112L}$ affording strain $\text{ZSC112G}$ (ptsG::cat $\text{glk manZ}$). Because the deletion of the already inactive ptsG allele in $\text{ZSC112L}$ cannot be confirmed by a phenotypic change, cat was also transduced in strain $\text{ZSC112G}$.

**Plasmid Constructions—** For the controlled expression of $\text{IIC}^{\text{Gle}}$ and of the IIB and IIC domains the vector pTSG11 with ptsG behind Ptac was constructed as follows. The HindIII/EcoRI fragment from plasmid pTSG4 (Erni and Zanolori, 1986) was inserted into the polylinker of the expression vector pF118EH (Furste et al., 1986). The resulting plasmid was linearized at the unique AatII site downstream of Ptac and upstream of the translation start codon, and digested with BstBI for different time intervals. The aliquots were digested with SmaI to remove the buffer sequence downstream of Ptac, and self-ligated. Strain $\text{ZSC112L}$ was transformed and colonies were gridded on two McConkey glucose plates containing no and 10 $\mu$M IPTG, respectively. Liquid cultures were grown from colonies which were strongly fermenting in the presence and weakly fermenting in the absence of IPTG. When the cultures had reached an $A_{550}$ of 0.5 they were divided into two aliquots of which one was induced with 0.1 mM IPTG. After 3 h the induced cells were precipitated with 15% trichloroacetic acid, washed with acetone, and the proteins were analyzed on an immunoblot with a monoclonal antiserum. The two putative $\sigma^A$ promoter sequences are underlined. Their homology scores calculated according to Mulligan et al. (1984) are 55 and 50%, respectively. $S/D$, putative ribosome binding site.

**Fig. 2. Plasmid maps of vectors used for domain expression and in vivo complementation experiments.** A, plasmid maps. Plasmid pTSG11 encodes $\text{IIC}^{\text{Gle}}$. Plasmids pQBH and pPBH encode the IIC$^{\text{Gle}}$-6H domain (pTSG390). Plasmids pACH and pPBH encode the IIBG$^{\text{Gle}}$-6H domain (pTSG390-6). Plasmid pABG421 encodes the IIC$^{\text{Gle}}$ DNA subunit with the C421S mutation (pTSG421). Plasmids pQBH, pPBH, and pPBH carry the ColEl replication origin (open arrow). Plasmids pABG421 and pPBH are derivatives of pACYC177 (Chang and Cohen, 1978) carrying the P15A origin of replication (open box), ara, amber mutation; bla, $\beta$-lactamase; cat, chloramphenicol acetyltransferase; kan, kanamycin resistance. Expression is constitutive from pPBH, pPBH, and pABG421 and under control of Ptac from pPBH and pPBH. B, 5'-noncoding nucleotide sequence of ptsG. The nucleotide to which the tacP promoter of the expression vector pF118EH (Furste et al., 1986) was fused is indicated with asterisks. The two putative $\sigma^A$ promoter sequences are underlined. Their homology scores calculated according to Mulligan et al. (1984) are 55 and 50%, respectively. S/D, putative ribosome binding site.
sus sequence of *ptg* (Fig. 2B).

Five additional plasmids which were constructed for this study by ligation of the appropriate restriction fragments from plasmids pACYC177 (Chang and Cohen, 1978), pTSG4-21 (Nuzzo et al., 1988), pTSG31 and pMC836 (Hummel et al., 1992), and pQEGH12 (Waeber et al., 1989) were used for expression in high-speed Bal31 exonuclease (3 units/4 pg DNA at 37 °C; Sambrook et al.) of strains ZSC112AG (pJCH) grown in LB broth. The cells were broken by two passages through a French pressure cell, cell debris was removed by a low-speed centrifugation (12,000 g; 4 °C; 10 min). Membranes were then solubilized by incubation at 37 °C for 1 h and further purified by gel filtration (Sephadex G-25) with buffers containing 50 mM NaF and 0.5% dodecylmaltoside. Membrane proteins were solubilized with 5% octyl-POE (BACHEM, Büren, Switzerland). The mixture was sonicated in a bath-type sonicator for 1 min, stirred for 15 min at 4 °C, and freed of nonsolubilized membranes by centrifugation (16,000 g; 4 °C; 1 h). The membranes were extracted with Low 4 × sonication in the same conditions. The collected supernatant (38 ml) was loaded on 3 ml of Ni2+-NTA resin equilibrated with buffer G (50 mM NaF, pH 8.0, 100 mM NaCl, 10 mM β-mercaptoethanol). Membrane proteins were eluted with 1 ml of buffer G and 15 ml of buffer H (50 mM NaF, pH 6.5, 100 mM NaCl, 10 mM β-mercaptoethanol, 1% deoxycholate, Sigma). Proteins were further purified by gel filtration (Superose 12, Pharmacia; 50 mM NaF, pH 7.5, 100 mM NaCl, 10 mM β-mercaptoethanol, 0.5% deoxycholate; flow rate 0.25 ml/min).

**Assay for Phosphoenolpyruvate:Phosphofructokinase Activity**

Sugar phosphorlyase activity was assayed by the ion exchange method of Kundig and Rosenman (1971) as described by Erni et al. (1997).

**Filter Binding Assay**—The assay is based on the quantitative binding of proteins to nitrocellulose filters. The assay mixture contained per 20 ml: 2.7 pmol of purified enzyme 1, 10 pmol of purified PPi, 0.4 pmol of purified IICα, 460 pmol of purified IICα-Glu, 50 mM NaF, pH 7.5, 10 mM MgCl2, 2.5 mM dithiothreitol, 2.5 mM NaF. The reaction was started by adding a 1.9 pmol of [32P]PPi (5–10 cpdpmol) and stopped at different time points by dilution of the reaction mixture with 1 ml of ice-cold buffer (10 mM NaF, pH 7, 150 mM NaCl). Proteins were adsorbed to cellulose nitrate filters (Sartorius) under suction. The filters were washed twice with 1 ml of buffer and counted in 6 ml of Insta-gel Plus (Packard). To measure the rate of protein dephosphorylation, IICα-Glu was phosphorylated for 15 min with [32P]PEP and then diluted with a 100-fold molar excess of unlabeled PEP ([32P]PEP was then diluted with a 100-fold molar excess of unlabeled PEP ([32P]PEP and stored at different temperatures. To terminate the reaction, 1 ml of ice-cold buffer (10 mM NaF, pH 7, 150 mM NaCl) was added and the samples were stored at −20 °C until assay. Assay mixtures were placed on phospho- and glycoprotein filters (Sartorius) and cyclic phosphorlyase activity was measured by the method of Kundig and Rosenman (1971) as described by Erni et al. (1997).

**Other Techniques**—All protein samples were boiled in sample buffer prior to electrophoresis. IICα-Glu was analyzed on standard 20% polyacrylamide gels (Erni et al., 1992). IICα-Glu was analyzed on standard 20% polyacrylamide gels (Erni et al., 1992). IICα-Glu was analyzed on standard 20% polyacrylamide gels.
plates and protein extracts were analyzed on immunoblots. The truncated IICBG" proteins of molecular masses between 10 and 16 kDa were found to be expressed in amounts varying by at least a factor of 10. Only the fragment with the highest expression level was further analyzed. This recombinant IIB domain (IIBG"-6H) contains residues 1-4 of the original IICBG" amino terminus followed by residues 391-476 of IICBG" and the hexahistidine tag at the carboxyl terminus (Fig. 3).

To express the IIC domain as a protein subunit, IICBG1'-6H was truncated after Arg346 in the LRTPRED sequence and fused to a hexahistidine tag (IICG1'-6H; Fig. 3). For in vivo complementation IICG1'-6H was expressed constitutively from the vector pACH (Fig. 2A) which is compatible with pB322-derived plasmids.

Purification of IIBG"-6H and IICG1'-6H—Plasmids pQBH and pICH were used (Fig. 2A) for expression and purification of IIBG"-6H and IICG1'-6H. As judged from in vitro activity tests the IPTG induced IIBG"-6H expression from pACH (Fig. 2A) is compatible with pB322-derived plasmids.

In Vivo Phosphotransferase Activity of IIBG"-6H and IICG1'-6H—The genes encoding IIBG"-6H and IICG1'-6H were cloned into compatible plasmids and expressed in a host strain (ZSC112AG). This strain carries a chromosomal deletion of ptsG (Erni and Zanolari, 1986). Unchanged amino acid sequences are omitted (dots).

The Domains of IICBG"c

**FIG. 3.** Characteristic amino acid sequence changes in the IICBG"c subunit and its two domains. Unchanged sequences are indicated in bold face. Additions and substitutions are indicated in italics. Residue numbers (superscripts) refer to the wild-type sequence (Erni and Zanolari, 1986). Unchanged amino acid sequences are omitted (dots).

**FIG. 4.** Purification of IIBG"c-6H. Coomassie (A) and alkaline silver (B)-stained polyacrylamide gels. 1, molecular weight markers; 2, cell-free, cytoplasmic extract; 3, IIBG"c-6H after Ni2+-NTA purification; 4, IIBG"c-6H after Sephadex 75 purification. 45 μg protein were loaded in lanes 1 and 2, 4.5 μg in panel B.

**FIG. 5.** Purification of IICG"c-6H. Coomassie Blue-stained polyacrylamide gel. 1, molecular weight markers; 2, cell-free membranes; 3, detergent-solubilized membrane proteins; 4, IICG"c-6H after Ni2+-NTA purification; 5, IICG"c-6H after Superose 12 purification. 20 μg of protein was loaded in lanes 2 and 3, 10 μg in lanes 4 and 5.

Tides; (ii) between IIBG"c-6H and the C421S mutant of the intact IICBG"c subunit (encoded by pABG421). IIBG"c-6H expression was varied by induction with IPTG. Results are summarized in Table III. The generation time is 125 min when expression of IIBG"c-6H is induced, and around 240 min when IIBG"c-6H is expressed from the uninduced leaky Ptac promoter. This is 1.5
IIBG" alone is unable to bind and phosphorylate the sugar between 50 and 95% of IIBG1'-6H could be labeled with 32P. It modulate the interaction between IIAGlc and IIBG1"; (ii) that equimolar amount substrate; (iii) that phospholipids and IIAGlc are necessary for (data not shown). The results shown in Fig. 6, faster rate than wild-type IICBG" (Fig. lane cate: (i) that IICG" does not participate in binding of IIAGlc nor (ii) that an phospholipids by decylmaltoside inhib- 

Activity was determined as aMG-6-P formed in the presence of

Substitution of phospholipids by decylmaltoside inhib-

Without plasmid >20 h ND a ND
IICBG(C421S) >20 h 125 48 0.93
IICBG1'-6H >20 h 125 48 0.93
IIBG1'-6H >20 h 225 60 0.93
Membranes 210 90 0.43 100 1
Membrane extract 152 72 0.39 80 0.93
Np-NTA 5.5 60 11.1 67 26
Superose 12 3.6 52 17.2 58 40.5

Purification of IIBG-C6-6H
Activity was determined as aMG-6-P formed in the presence of PEP, EI, HPr, IIAGlc, and membranes containing IIAGlc-6H.

and 3 times longer than the generation time of the wild-type control. IIBG1'-6H complements both IIAGlc-6H and the IICBG" mutant with an inactive IIB (C421S) domain with equal efficiency. These observations suggest that: (i) binding of IIBG" to the IIC domain is likely to be rate-limiting and (ii) that an inactive IIB domain covalently bound to IIC does not sterically or otherwise inhibit the binding of an active IIB domain.

In Vitro Activity of the Purified IIB and IIC Domains—Two functional properties are measured: (i) the phosphorylation of IIBG1'-6H by IIAGlc with IIB serving as a substrate, and (ii) the phosphorylation of glucose with IIBG1'-6H and IIAGlc-6H serving as catalysts. Isolated IIBG1'-6H is phosphorylated by IIAGlc in the presence of enzyme I and HPr (Fig. 6A, lanes 1–5). IIBG" is not dephosphorylated by glucose alone (Fig. 6A, lane 6), but is dephosphorylated completely upon addition of glucose together with the IICG"-6H domain and E. coli phospholipids (Fig. 6A, lane 8). Substitution of phospholipids by decylmaltoside inhibits the IICG"-6H domain obviously does not prevent independent hydrolysis of the phosphothioester (Fig. 6C). It is 40 h for IIBG"-6H and 9 h for wild-type IICBG"-6H.

The sugar phosphorylation activity of IIB and IIC were determined in the standard sugar phosphotransferase assay. IIBG1'-6H and IIAGlc-6H complement each other. Phosphorylation of methyl-α-D-glucopyranoside depends on the concentration of both IIBG1'-6H and IIAGlc-6H (Fig. 7). The specific activity of IIAGlc-6H (19 nm) in the presence of IIBG1'-6H (1.9 μM) is 2% of IIAGlc wild-type activity. In spite of the 100-fold excess of IIBG1'-6H over IIAGlc-6H the IIC activity is not saturated.

DISCUSSION

The transporters of the bacterial phosphotransferase system consist of three functional units (IJA, IIB, and IIC) that are either protein subunits in a complex or domains of a multidomain protein. It is not clear when and why the two apparently equivalent forms are implemented. The E. coli transporters for mannitol (Lee and Saier, 1983) and GlcNac (Peri and Waygood, 1988) and the B. subtilis transporter for glucose (Sutrina et al., 1990; Zagorec and Postma, 1992) are three-domain proteins (ICBA). In contrast, the cellulose transporters of E. coli (Reizer et al., 1990) and Bacillus steaerothermophilus (Lai and Ingram, 1993) are complexes of three subunits (IJA, IIB, and IIC). The E. coli transporter for glucose consists of two subunits IIAGlc and IICBG. The two domains of the IICBG subunit, IIBG-C6-6H and IICBG-C6-6H, have been isolated and purified to homogeneity by Ni2+ chelate affinity chromatography. IIBG1'-6H is able to restore the in vivo and in vitro activities of a IICBG(C421S) mutant as well as of the separated IICG"-6H domain. An inactive IIB domain although covalently attached to the IIC domain obviously does not prevent independent IIBG" moieties from interacting with IIC. Such a loose connection between the two domains would not have been predicted from the linker sequence which is as strongly conserved between the three homologous transporters, IICBG, IICBG(C6)-Nac (Peri and Waygood, 1988), and MaX (Reidl and Boos, 1991), as
the sequence including the active site cysteine. Usually, protein linkers are characterized by their amino acid composition rather than by a particular sequence (Erni, 1989; Wootten and Drummond, 1989; Argos, 1990; Perham, 1991). The IIA^c subunit allosterically activates the phosphotransfer reaction from phospho-IIIC^c-glucose to ErnI (Erni, 1986) and allosteric interactions between the IIB and IIC domains are likely to play a role in translocation of glucose. In contrast, the rate of phosphoryltransfer from the IIA^c-glucose to the IIB^c domain is not affected by the absence of the IIC domain, suggesting that the property of IIB as phospholipasec is not altered by IIC. The overall rate of phosphoryltransfer from IIA^c-glucose to the sugar substrate is, however, considerably slower than in the wild-type IIBC^c-glucose. Separation of IIB and IIC results in an overall 1.5-fold increase of generation time and a 50-fold decrease of in vitro activity. Different IIB^c-glucose concentrations can account for the discrepancy between in vivo and in vitro activity. The estimated in vivo concentration of IIB^c-glucose is 30-fold higher than the concentration that has been used in vitro.

A number of successful gene dissection and gene reconstruction experiments have been reported for other phosphoenolpyruvate-sugar phosphotransferase system transporters. The separate IIA^m subunit domain is able to restore the activity of the IICBAM^h554A mutant to 25% of wild-type activity with an apparent K_0 of 26 µM (van Weeghel et al., 1991a). The separated B. subtilis IIA^c restored to 100% and with a K_0 of 25 µM for the E. coli IICB^c activity (Reizer et al., 1992). The IIA^m domain of the E. coli transporter for mannose was subcloned and characterized (Erni et al., 1989). In all cases subcloning allowed the purification of enough protein for structure determination by x-ray and NMR techniques (Génonvès-Taverne et al., 1990; Fairbrother et al., 1991a, 1991b, 1992; Liao et al., 1991; Lammers et al., 1992; Chen et al., 1993a, 1993b; Kroon et al., 1993; Seip et al., 1994). More pertinent to our investigation is the separate expression and purification of the IIIC^m subcomplex and of the IIBC^m domain of the mannitol transporter by Robillard and co-workers (1993). The activity of IIB^m combined with a membrane preparation containing the isolated IIC domain is 2% at a IIB^m/IIC^m ratio of 70 and 9% at a ratio of 5000 (van Weeghel et al., 1991b). The combined activity of the isolated IIA, IIB, and IIC domains of the mannitol transporter is 4% (Robillard et al., 1993). The 2% combined activity obtained with IIB^c-glucose and IIC^c-glucose is thus of the same order. The optimal function of the IIB and IIC domains appears to depend more critically on the covalent interdomain link than the interactions between the IIA and IIB domains. Although no systematic studies have been performed two examples might illustrate how the association of IIB with IIC must be much tighter than of IIB with IIA. The A-B interaction between wild-type IIA^c-glucose and IIBC^c-glucose has a K_0 of 1.7 µM (Reizer et al., 1992). However, their activity can be increased 4-fold when these naturally separate subunits are fused into a single polypeptide chain (Schunk et al., 1992). The B-C interaction between the naturally separate wild-type IIA^m IB and the IIC^m complex of the mannose transporter has a K_0 of 5–10 nm. If the B-C interaction has to be so tight it is not

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Footnote: 3 B. Erni, unpublished results.
surprising that naturally linked IIB and IIC domains lose activity when the covalent link is cleaved. By selecting mutants with shorter generation times it should be possible in the future to identify mutations of IIB and/or IIC with increased affinity for each other. The biochemical and molecular characterization of such mutants might reveal residues at the protein-protein interface which are critical for binding of IIC and IIB in the IIC\textsuperscript{Glc} subunit.

No assumptions were made as to where the IIB\textsuperscript{Glc} domain exactly begins. Instead IIC\textsuperscript{Glc} was truncated progressively from the NH\textsubscript{2} terminus toward the COOH terminus and the most stable form was identified by screening for protein expression and activity. This optimization of protein stability and the use of Ni\textsuperscript{2+} chelate affinity chromatography allowed the purification of 40 mg of pure IIB\textsuperscript{Glc}-6His from 1 liter of cell culture. The IIB domain thus identified starts close to the LKTPGRED sequence which was proposed to serve as a hinge between the IIC and IIB domains in the transporters for glucose and GlcNAc (Hummel et al., 1992). This sequence also forms the fusion joint of the only biologically active IIC\textsuperscript{Glc}::IIB\textsuperscript{Glc} hybrid protein found so far. When using homologous recombination instead of site-directed mutagenesis to fuse nAGE and pTSG, only those IIC\textsuperscript{Glc}::IIB\textsuperscript{Glc} hybrids with the fusion join in the LKTPGRED sequence turned out to be active.\textsuperscript{3}

Based on this work the secondary structure of IIB\textsuperscript{Glc}-6His has been assigned by heteronuclear NMR spectroscopy (Golic Grdadolinik et al., 1994). The long term stability of phosphorylated IIB\textsuperscript{Glc}-6His makes NMR experiments with phosphorylated IIB\textsuperscript{Glc}-6His feasible. Structural refinements and NMR studies of the phosphorylated form of the IIB\textsuperscript{Glc} domain are in progress.

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