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The *Escherichia coli* glucose transporter enzyme IICB^{Glc} recruits the global repressor Mlc

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In addition to effecting the catalysis of sugar uptake, the bacterial phosphoenolpyruvate:sugar phosphotransferase system regulates a variety of physiological processes. Exposure of cells to glucose can result in repression or induction of gene expression. While the mechanism for carbon catabolite repression by glucose was well documented, that for glucose induction was not clearly understood in *Escherichia coli*. Recently, glucose induction of several *E.coli* genes has been shown to be mediated by the global repressor Mlc. Here, we elucidate a general mechanism for glucose induction of gene expression in *E.coli*, revealing a novel type of regulatory circuit for gene expression mediated by the phosphorylation state-dependent interaction of a membrane-bound protein with a repressor. The dephospho-form of enzyme IICB^{Glc}, but not its phospho-form, interacts directly with Mlc and induces transcription of Mlc-regulated genes by displacing Mlc from its target sequences. Therefore, the glucose induction of Mlc-regulated genes is caused by dephosphorylation of the membrane-bound transporter enzyme IICB^{Glc}, which directly recruits Mlc to derepress its regulon.

Keywords: enzyme IICB^{Glc}/glucose induction/Mlc/protein–protein interaction/signal transduction

Introduction

Sensory transduction is an important device for monitoring the environment of all organisms. Bacteria sense continuous changes in their environment and adapt metabolically to compete effectively with other organisms for limiting nutrients. One system that plays an important part in this adaptation response is the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The PTS is composed of two general cytoplasmic proteins, enzyme I (EI) and histidine phosphocarrier protein HPr, which are used for all sugars, and, in addition, some sugar-specific components collectively known as enzymes II (Postma *et al*., 1996). The primary function of this multifunctional system is the concomitant phosphorylation and translocation of its numerous sugar substrates across the cytoplasmic membrane. Glucose-specific enzyme II of *Escherichia coli* consists of two subunits: soluble enzyme IIA^{Glc} (EIIA^{Glc}) and membrane-bound enzyme IICB^{Glc} (EIICB^{Glc}). Thus, glucose transport in *E.coli* involves three soluble PTS components (EI, HPr and EIIA^{Glc}, encoded by the *ptsHIcrr* operon) and one membrane-bound protein, enzyme IICB^{Glc} (encoded by the *ptsG* gene). Glucose uptake entails sequential phosphoryl transfer via the PTS, as follows: phosphoenolpyruvate (PEP) ⇒ EI ⇒ HPr ⇒ EIIA^{Glc} ⇒ EIICB^{Glc} ⇒ glucose.

In addition to its sugar transport activities, the PTS takes part in a variety of physiological processes, including chemoreception (Lux *et al*., 1995), catabolite repression (Stülke and Hillen, 1999), carbohydrate transport and metabolism (Postma *et al*., 1996; Seok *et al*., 1997b), carbon storage (Seok *et al*., 1997a), and the coordination of carbon and nitrogen metabolism (Powell *et al*., 1995). The regulatory functions of the PTS depend on the phosphorylation state of the involved component, which increases in the absence and decreases in the presence of a PTS sugar substrate. The ratio of phosphorylated to dephosphorylated proteins in turn serves as signal input for the control of these physiological processes.

As expected for a system as central to bacterial metabolism as the PTS, synthesis of the PTS proteins is regulated in a highly sophisticated way. In enteric bacteria, expression of the *pts* operon increases during growth on glucose and requires an intact cAMP–cAMP receptor protein (CRP) system, although growth on glucose reduces the concentration of cAMP and CRP in the cell (Postma *et al*., 1993). While the mechanism for carbon catabolite repression by glucose is well understood (Postma *et al*., 1996; Stülke and Hillen, 1999), that for glucose induction was not clarified in *E.coli*. Glucose mediates transcriptional activation of several genes for PTS-related sugar transporters and some enzymes involved in glycolysis (Postma *et al*., 1996; Charpentier *et al*., 1998). Recently, glucose induction of several PTS operons and other genes was shown to be mediated by the Mlc (making large colonies) protein (Hosono *et al*., 1995). The *mlc* gene was originally found to cause the reduction of acetate accumulation when overexpressing cells grow in the presence of glucose. The *mlc* gene was shown to be identical to the previously characterized *dgsA* gene (Roehl and Vinopal, 1980; Morris *et al*., 1985; Plumbridge, 1998a). The Mlc protein (44 kDa) has been shown to act as a repressor for several catabolic operons whose products are linked to sugar metabolism. These Mlc-regulated genes include the *manXYZ* operon encoding the mannose PTS (Plumbridge, 1998a), the *malT* gene encoding the transcriptional

activator of the maltose regulon (Decker *et al*., 1998), the *ptsG* gene (Kimata *et al*., 1998; Plumbridge, 1998b) and the *ptsHIcrr* operon (Kim *et al*., 1999; Plumbridge, 1999; Tanaka *et al*., 1999) as well as the *mlc* gene itself (Decker *et al*., 1998). A common feature of the five operons thus far identified as members of the Mlc regulon is that they possess at least one cAMP–CRP binding site as well as an Mlc binding site, and thus are under dual regulation. This seems to be necessary for the intricate regulation of expression of these genes in order to respond flexibly to various environmental conditions.

Although Mlc was proven to be a mediator of glucose induction of several genes, the mechanism of this mediation was not understood. In this report, we describe a general mechanism for glucose induction of gene expression in *E.coli*. We provide experimental evidence showing that glucose induction is mediated through the direct physical interaction between Mlc and EIICB^{Glc}, dependent on the phosphorylation state of EIICB^{Glc}.

Results

Overproduction of EIICB^{Glc} induces transcription of Mlc-regulated genes in vivo

The activities of several transcription factors are regulated by interaction with other proteins, for example, the regulation of several sigma factors by their anti-sigma factors (Helman, 1999; Kang *et al*., 1999). Since it is also well established that PTS proteins interact with and regulate several proteins (Postma *et al*., 1996; Seok *et al*., 1997a), we addressed the possibility that glucose induction might be mediated by a protein–protein interaction involving the PTS, as proposed previously (Kim *et al*., 1999; Plumbridge, 1999). If this were the case, overexpression of the partner protein interacting with Mlc should induce expression of the Mlc regulon by sequestration of the repressor. We examined this question using *E.coli* strain GI698 (LaVallie *et al*., 1993; Seok *et al*., 1996) transformed with pRE1-based recombinant plasmids constructed for overexpression of the four PTS proteins necessary for glucose uptake. In plasmids pRE1-*ptsG* and pRE1-*ptsHIcrr* used in this work, genes are under the control of the strong λP_L promoter–cII ribosome binding site combination (Reddy *et al*., 1989).

The level of transcription from the two Mlc-regulated operons on the chromosome, *ptsHIcrr* and *ptsG*, was examined in different cells (Figure 1). The transcripts from the plasmid genes were selectively excluded by using a probe specific for the untranslated region of the chromosomal gene absent in the recombinant plasmids. While overproduction of three soluble PTS proteins (EI, HPr and EIIA^{Glc}) did not change the level of the endogenous *ptsG* transcript (lanes labeled pRE1-*ptsHIcrr*), it was increased by the overproduction of EIICB^{Glc} (lanes labeled pRE1-*ptsG*) (Figure 1A). Thus, EIICB^{Glc} exerts a strong stimulatory effect on *ptsG* expression that was not observed with EI, HPr and EIIA^{Glc}.

The effect of EIICB^{Glc} depletion or overproduction on another Mlc-regulated operon (*ptsHIcrr*) was also examined. Primer extension analysis of the *ptsHIcrr* transcripts demonstrated that the lack of EIICB^{Glc} (compare lanes labeled GI698 with lanes labeled GI698*ΔptsG*) caused a reduction in the level of the *pts* P0 transcript (see arrowhead, Figure 1B) whereas overproduction of EIICB^{Glc} (lanes labeled GI698/pRE1-*ptsG*) increased the level, even in the absence of glucose (Figure 1B). In all cases, the *pts* P0 transcript was increased by glucose. These results imply that dephosphorylated EIICB^{Glc} in the membrane, but not the soluble PTS proteins, may act as a positive regulator of the Mlc regulon, possibly by neutralizing Mlc activity. A previous report arguing that the signal for the induction of *pts* gene expression might be an increase in the amount of unphosphorylated EIICB^{Glc} is consistent with these data (De Reuse and Danchin, 1991).

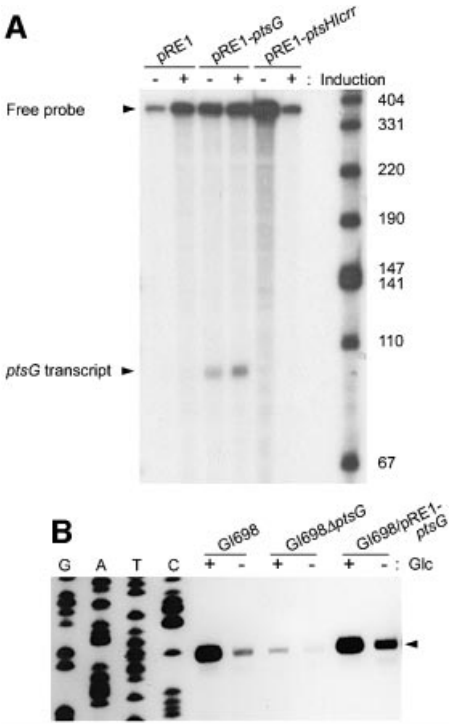

Fig. 1. Overproduced EIICB^{Glc} induces the expression of genes encoding PTS proteins *in vivo*. (**A**) S1 nuclease protection analysis of *ptsG* transcripts. RNA samples prepared from GI698 cells harboring the indicated plasmids grown with and without induction of protein expression were hybridized with the ^{32}P-labeled probe and digested with S1 nuclease as described in Materials and methods. S1 mapping analysis was accomplished four times and representative data are shown. The transcriptional level of Mlc-regulated *ptsG* increased in GI698 cells harboring pRE1-*ptsG* after induction of EIICB^{Glc} production. (**B**) Effect of EIICB^{Glc} on transcription of the *ptsHIcrr* operon analyzed by primer extension analysis. RNA samples were prepared from the indicated strains grown in tryptone broth with or without glucose (Glc). The arrowhead indicates the *pts* P0 transcripts.

Direct interaction between Mlc and EIICB^{Glc}

We then examined whether purified Mlc interacts directly with the purified PTS components, EI, HPr and EIIA^{Glc}. As expected from the data in Figure 1, real-time interaction analyses using BIAcore and electrophoretic mobility shift assays with purified proteins did not show any interaction between Mlc and the soluble PTS components (EI, HPr and EIIA^{Glc}) regardless of their phosphorylation states (data not shown). Thus, we explored the interaction between Mlc and EIICB^{Glc} in the form of membrane vesicles. EIICB^{Glc}-depleted and -enriched membrane vesicles were prepared from *E.coli* GI698*ΔptsG* cells transformed with pRE1 and pRE1-*ptsG*, respectively. PEP phosphorylated PTS components including membrane-bound EIICB^{Glc}, while

glucose completely dephosphorylated those proteins. No Mlc was co-pelleted with the membrane vesicles prepared from strain GI698*ΔptsG* transformed with the control vector pRE1, regardless of the phosphorylation state of the added soluble PTS proteins (Figure 2, lanes labeled –EIICB^{Glc}). However, when glucose was added to the reaction mixture containing EIICB^{Glc}-enriched membrane vesicles, Mlc was completely co-precipitated with the membrane vesicles and no Mlc was detected in the supernatant (compare lanes for supernatants in Figure 2). When PEP was added to a reaction mixture containing EIICB^{Glc}-enriched membrane vesicles and the three soluble PTS proteins, most of the added Mlc was partitioned into the soluble fraction. Therefore, the binding of Mlc to EIICB^{Glc} occurred in a phosphorylation-dependent manner. These results provide strong evidence that there is a direct physical interaction between the membrane-bound glucose transporter EIICB^{Glc} and the global repressor Mlc, and that this interaction is dependent on the availability of glucose and other PTS proteins that can dephosphorylate EIICB^{Glc} concomitant with glucose transport. It should be noted, however, that EIICB^{Glc}-enriched membrane vesicles prepared from GI698*ΔptsHIcrr* interacted with Mlc even in the absence of glucose, implying that the direct regulator for the Mlc–EIICB^{Glc} interaction is not glucose but the phosphorylation state of EIICB^{Glc} (data not shown). This also implies that the soluble PTS proteins are not involved in the interaction.

The soluble EIIB domain is sufficient for the phosphorylation state-dependent interaction with Mlc

Since the interaction between Mlc and EIICB^{Glc} was only dependent on the phosphorylation state of EIICB^{Glc}, we tested the possibility that the isolated EIIB domain is sufficient for the interaction with Mlc in a phosphorylation state-dependent manner. Consequently, we cloned plasmid pJHK (see Materials and methods) to overexpress EIIB by removing the linker and the IIC domain from EIICB^{Glc}. The purified EIIB domain could be phosphorylated by sequential phosphoryl transfer from PEP via EI, HPr and EIIA^{Glc} (Figure 3A). Thus, we purified to homogeneity the phosphorylated EIIB by employing FPLC Mono Q 5/5 column chromatography, and mixed it with unphosphorylated EIIA^{Glc}. The phosphoryl transfer reaction between EIIB and EIIA^{Glc} was shown to be reversible (Figure 3B).

The direct phosphorylation state-dependent interaction of EIIB and Mlc was demonstrated by surface plasmon resonance (SPR) using a BIAcore optical biosensor (Seok *et al*., 1997a; Kang *et al*., 1999). When purified Mlc was exposed to immobilized EIIB, high affinity interaction was detected (Figure 3C, left sensorgram). In contrast, after the immobilized EIIB domain was phosphorylated by flowing the mixture of EI, HPr and EIIA^{Glc} in the presence of PEP, and subsequently washed with the running buffer, interaction of Mlc was hardly detectable (center sensorgram). Furthermore, the immobilized EIIB domain recovered Mlc binding activity after flowing dephosphorylated EIIA^{Glc} through the flow cell and flushing with the running buffer (Figure 3C, right sensorgram). These results provide direct evidence for the interaction between EIICB^{Glc} and Mlc, and indicate that the EIIB domain of EIICB^{Glc} is sufficient for the phosphorylation state-dependent interaction with Mlc.

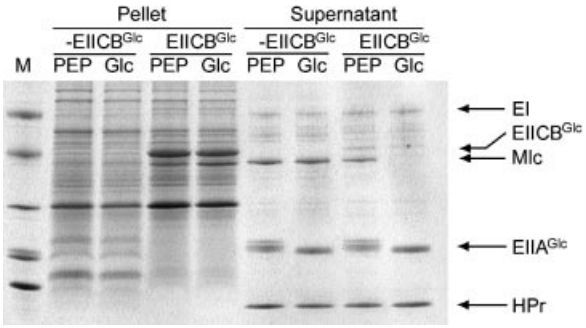

Fig. 2. Direct interaction between the dephospho-form of EIICB^{Glc} and Mlc. Membrane vesicles (350 μg) in which EIICB^{Glc} is enriched (lanes labeled EIICB^{Glc}) or EIICB^{Glc} is lacking (lanes labeled –EIICB^{Glc}) were mixed with Mlc and three soluble PTS proteins (EI, HPr and EIIA^{Glc}) in the presence of 2 mM PEP or 2 mM glucose (Glc), as indicated. After incubation for 5 min, EIICB^{Glc}-bound proteins were separated by pelleting membrane vesicles using an Airfuge. The supernatants and pellets were run on SDS–PAGE gels (see Materials and methods). M, Mark 12™ protein standard (Novex).

Binding affinity of Mlc with EIICB^{Glc} and its target promoters

To determine the strength of the EIICB^{Glc}–Mlc interaction, we incubated Mlc with various concentrations of EIICB^{Glc} in the form of membrane vesicles. Then we estimated the amount of free Mlc, remaining soluble after pelleting membrane vesicles and proteins bound to them, by densitometric quantification of the Mlc bands in SDS–PAGE (Figure 4). As the amount of added EIICB^{Glc} increased, that of Mlc in the soluble fractions decreased, while the level of the added soluble PTS proteins remained constant. This also supports the premise that Mlc interacts directly with EIICB^{Glc} without involving any soluble PTS proteins. Recently, single particle analysis of EIICB^{Glc} in proteoliposomes showed it to be a dimer (Zhuang *et al*., 1999), whereas gel filtration chromatography of the purified Mlc reveals its molecular weight to be ~172 kDa, indicating that Mlc is a tetramer (T.-W.Nam and Y.-J.Seok, unpublished data). From the titration data in Figure 4A, the dissociation constant (K_d) was calculated to be ~10^{-7} M, assuming interaction between a tetrameric form of Mlc and a dimeric form of EIICB^{Glc}.

The strength of the interaction between the dephospho-form of EIICB^{Glc} and Mlc was compared with that of the interaction between Mlc and its target DNA sites, in order to obtain a perspective on whether the dephospho-form of EIICB^{Glc} could sequester Mlc from binding to the operator sites in the target promoters. The dissociation constants between Mlc and its target promoters were measured by gel shift assays (Figure 4B). The K_d of the interaction between the *pts* P0 promoter and Mlc (Figure 4B, upper panel) was ~10^{-8} M, which is ~10-fold lower than the K_d between Mlc and EIICB^{Glc}. The K_d of the interaction between Mlc and the *ptsG* promoter was almost the same as that of the Mlc–*pts* P0 interaction (data not shown), while the strength of the interaction between Mlc and the *mlc* promoter was ~10 times weaker (K_d of ~10^{-7} M) (Figure 4B, lower panel). Since the intracellular level of

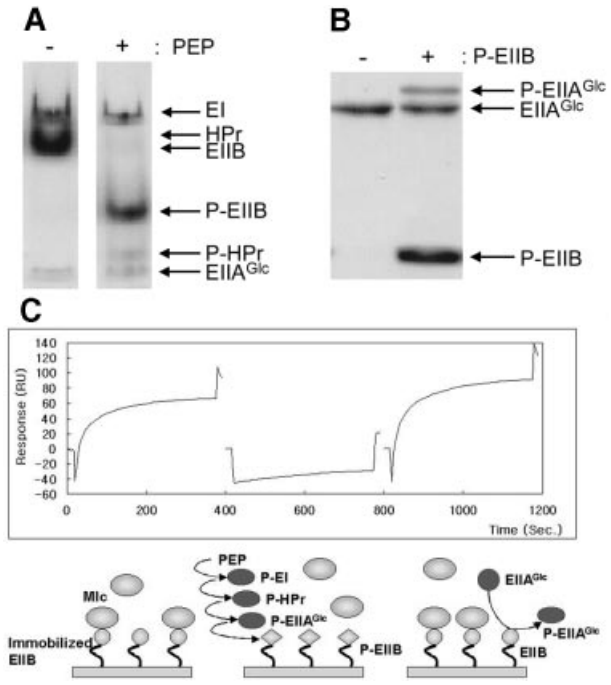

Fig. 3. The EIIB domain interacts with Mlc in a phosphorylation state-dependent manner. (**A**) The EIIB domain of EIICB^{Glc} can be phosphorylated by EIIA^{Glc} in the presence of PEP, EI and HPr. EIIB (3 μg) was incubated with EI, HPr and EIIA^{Glc} (1 μg each) in the absence (–) or presence (+) of 1 mM PEP in the reaction buffer (10 mM Tris–HCl pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA and 1 mM DTT) at room temperature for 5 min, and run on a 15% polyacrylamide gel under non-denaturing conditions. The gel was stained with Coomassie Blue. (**B**) Reversible phosphotransfer reaction between EIIB and EIIA^{Glc}. Phosphorylated EIIB was purified and incubated with unphosphorylated EIIA^{Glc} in the reaction buffer described in (A). After incubation at room temperature for 5 min, the mixture was examined by SDS–PAGE using a 15% polyacrylamide gel. The gel was stained with Coomassie Blue. (**C**) Phosphorylation state-dependent interaction between EIIB and Mlc measured by changes in SPR. Purified EIIB was immobilized on the carboxymethylated dextran surface of a CM5 sensor chip. Mlc (20 μg/ml) was allowed to flow over the EIIB surface for 6 min in each sensorgram. The phospho- and dephospho-EIIB surfaces were generated by the reversible phosphoryl transfer reactions between EIIB and EIIA^{Glc}, as described in Materials and methods, and are shown schematically below each sensorgram. The first sensorgram shows Mlc binding to the immobilized EIIB surface without any treatment. In the second sensorgram, Mlc was injected after the immobilized EIIB surface had been phosphorylated by flowing the mixture of EI, HPr and EIIA^{Glc} in the presence of PEP, and flushed with the running buffer to remove PEP and other PTS proteins. In the third sensorgram, dephosphorylated EIIA^{Glc} was allowed to flow over the phospho-EIIB surface generated in the second sensorgram to dephosphorylate it before Mlc was injected.

EIICB^{Glc} is at least 100 times higher than that of Mlc and its target promoters (Kimata *et al*., 1998; Kim *et al*., 1999; Rohwer *et al*., 2000), and the K_d of the interaction between Mlc and the *pts* P0 and *ptsG* promoters is only ~10-fold lower than that between EIICB^{Glc} and Mlc, it is reasonable to assume that Mlc could be completely displaced from its target DNA sites by interacting with EIICB^{Glc} under physiological conditions in the presence of glucose in the medium.

The dephospho-form of EIICB^{Glc} displaces Mlc from its target promoters to induce transcription in vitro

To support the idea that EIICB^{Glc} induces the Mlc regulon by interacting with and displacing Mlc from its target

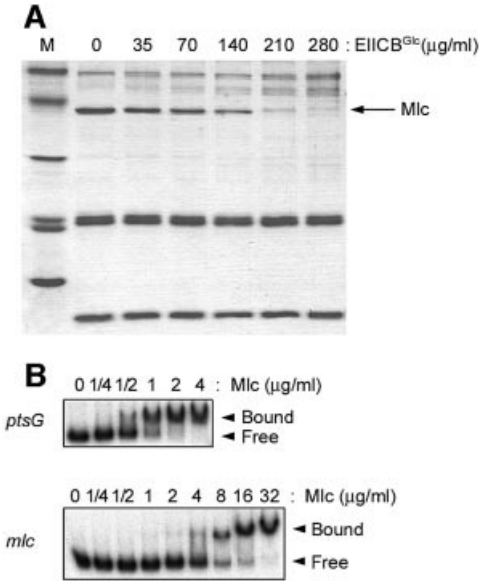

Fig. 4. Determination of the binding affinity of Mlc interaction with EIICB^{Glc} and its target sequences of the Mlc regulon. (**A**) Interaction between dephosphorylated EIICB^{Glc} and Mlc. Following incubation of Mlc (100 μg/ml) with the indicated concentrations of membrane-bound EIICB^{Glc} in the presence of EI, HPr, EIIA^{Glc} and 2 mM glucose, the reaction mixtures were centrifuged to separate Mlc bound to EIICB^{Glc} in membrane vesicles. The supernatant fractions were electrophoresed by SDS–PAGE and stained with Coomassie Blue. The calculated K_d of the interaction between EIICB^{Glc} and Mlc was ~10^{-7} M. (**B**) Interaction between Mlc and its target sites on the *ptsG* and *mlc* promoters. ^{32}P-labeled DNA probe (600 ng/ml) was mixed with the indicated concentrations of Mlc and then electrophoresed on 6% polyacrylamide gels. The calculated K_d of the interaction was ~10^{-8} M between Mlc and the *ptsG* promoter, and ~10^{-7} M between Mlc and the *mlc* promoter.

DNA in the presence of glucose, the effect of EIICB^{Glc} on the binding of Mlc to its target sites on the *pts* P0, *ptsG* and *mlc* promoters was examined by gel shift assays and *in vitro* transcription analysis (Figure 5). The addition of EIICB^{Glc}-enriched membrane vesicles to the reaction mixture inhibited the binding of Mlc to its target sites on the *pts* P0, *ptsG* and *mlc* promoters in the presence of glucose, whereas EIICB^{Glc}-deficient membrane vesicles did not inhibit the binding of Mlc (lanes 1–3 and 7–9, Figure 5A; data not shown for the *mlc* promoter). EIICB^{Glc}-enriched membrane vesicles incubated with Mlc under phosphorylating conditions (in the presence of PEP and the three purified soluble PTS proteins) exerted little effect on the binding activity of Mlc to its target DNA (lanes 4–6). Displacement of Mlc by the dephospho-form of EIICB^{Glc} from its target site was also verified by *in vitro* transcription assays. Using a supercoiled plasmid containing the *ptsHIcrr* promoter region, we recently showed that Mlc selectively repressed activity of the P0 promoter but not that of the P1 promoter (Kim *et al*., 1999). As shown in Figure 5B, EIICB^{Glc} dephosphorylated by adding glucose could relieve repression of the *pts* P0 transcription by Mlc, whereas the phosphorylated form of EIICB^{Glc} did not affect Mlc action. Neither EIICB^{Glc} nor Mlc had any effect on transcription from the P1 promoter, indicating that EIICB^{Glc} specifically relieves Mlc-dependent repression. These results indicate that glucose-dependent dephosphorylation enables EIICB^{Glc} to interact tightly with Mlc, resulting in sequestration of the repressor from its

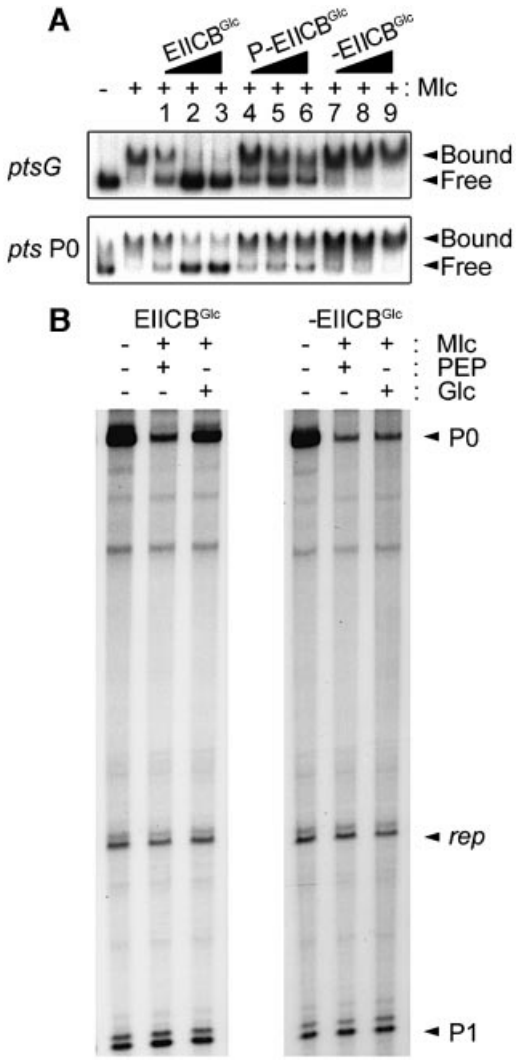

Fig. 5. The dephospho-form of EIICB^{Glc} displaces Mlc from its target DNA to induce transcription. (**A**) Inhibition of Mlc binding to its target DNA by dephosphorylated EIICB^{Glc}. ^{32}P-labeled promoter DNA probes [*ptsG* (upper panel) and *pts* P0 (lower panel)] were mixed with 1 μg/ml Mlc and membrane vesicles in the binding buffer described in Figure 4. Membrane-bound EIICB^{Glc} in the reaction mixture was phosphorylated with 2 mM PEP (lanes labeled P-EIICB^{Glc}) and dephosphorylated with 2 mM glucose (lanes labeled EIICB^{Glc}). Only dephosphorylated EIICB^{Glc} efficiently displaced Mlc from its target DNA. Membrane vesicles lacking EIICB^{Glc} were used as control (lanes labeled –EIICB^{Glc}). Membrane vesicles were at 3.3 μg/ml (lanes 1, 4 and 7), 10 μg/ml (lanes 2, 5 and 8) and 30 μg/ml (lanes 3, 6 and 9). (**B**) Induction of *pts* P0 transcription by EIICB^{Glc} in the presence of glucose *in vitro*. The *ptsHIcrr* promoter on the supercoiled plasmid pHX was used for *in vitro* transcription assays (Kim *et al*., 1999). A 185 nucleotide transcript from P0 and an 85 nucleotide transcript from P1 promoters are indicated by arrowheads. The transcripts from the plasmid origin of replication (106/107 nucleotides) are marked as *rep*.

binding sites in the target promoters, thereby derepressing gene expression in response to glucose.

Discussion

Glucose induces expression of several genes, many of which have been shown to be under the control of Mlc in *E.coli*. The inducing signal between glucose and Mlc, however, had not previously been identified. Several possibilities have been considered for the mechanism of glucose induction of the Mlc regulon. For many transcriptional repressors of specific operons for sugar transport, the inducer is a low-molecular-weight metabolite related to the transported substrate. Since the sequence of Mlc is ~40% identical to NagC, a repressor and activator of the genes encoding enzymes for amino sugar degradation in response to *N*-acetylglucosamine-6-phosphate (Plumbridge, 1998a), the possibility that glucose or one of its metabolic intermediates may act as the inducer of Mlc-regulated genes has been considered. However, no simple sugars or their metabolic intermediates have been found to inactivate Mlc *in vitro* (Plumbridge, 1998b; Kim *et al*., 1999).

Regulation of the activities of several transcription factors has been reported to be mediated by direct phosphorylation. Many response regulators of the two-component signal transduction systems act as transcription factors whose activities are regulated by signal-dependent phosphorylation (Hoch, 2000). Transcriptional regulation of the *bgl* operon in *E.coli* was also shown to involve phosphotransferase system-mediated phosphorylation of BglG, a transcriptional antiterminator and the response regulator of the *bgl* sensory system (Amster-Choder and Wright, 1997; Chen *et al*., 1997). De Reuse and Danchin (1991) previously proposed a signal transduction mechanism to explain how EIICB^{Glc} induced *ptsHIcrr* expression in response to glucose, based on the weak homology between the C-terminal EIIB domain of EIICB^{Glc} and the sensor kinases of the two-component signal transduction systems. Although Mlc did not exhibit any homology to known response regulators, it might be imagined that PTS-mediated phosphorylation of Mlc would change its activity, since both the repression of several genes by Mlc and the phosphorylation of PTS proteins are dependent on the presence of its sugar substrate. This possibility, however, has been ruled out by our previous experiments (Kim *et al*., 1999).

Several other transcription factors are regulated by the interaction with their partner proteins, as demonstrated for the regulation of several sigma factors by their anti-sigma factors (Helman, 1999). In *Bacillus subtilis*, CcpA, the repressor/activator mediating carbon catabolite repression and glucose activation, was reported to form a complex with seryl-phosphorylated HPr for regulation of the *pta* gene (Presecan-Siedal *et al*., 1999). There are also a few reports indicating the involvement of membrane-bound or membrane-associated proteins in controlling the activity of transcription factors in *E.coli*. A recent report proposed that the RpoE heat-shock sigma factor is modulated by physical interaction with the membrane-bound anti-sigma factor RseA, although the mechanism for the regulation of the interaction was not clearly elucidated (Missiakas *et al*., 1997). It has also been demonstrated that MalK, a component of the ABC-type transporter for maltose, physically interacts with MalT to downregulate its transcriptional activity in response to the levels of ATP hydrolysis associated with maltose transport (Panagiotidis *et al*., 1998). Thus, we considered the protein–protein interaction as a reasonable mechanism for glucose induction of the Mlc regulon. If one of the PTS proteins interacted with Mlc to mediate signal transduction between glucose and Mlc, overproduction of the PTS proteins should increase both their phospho- and dephospho-forms,

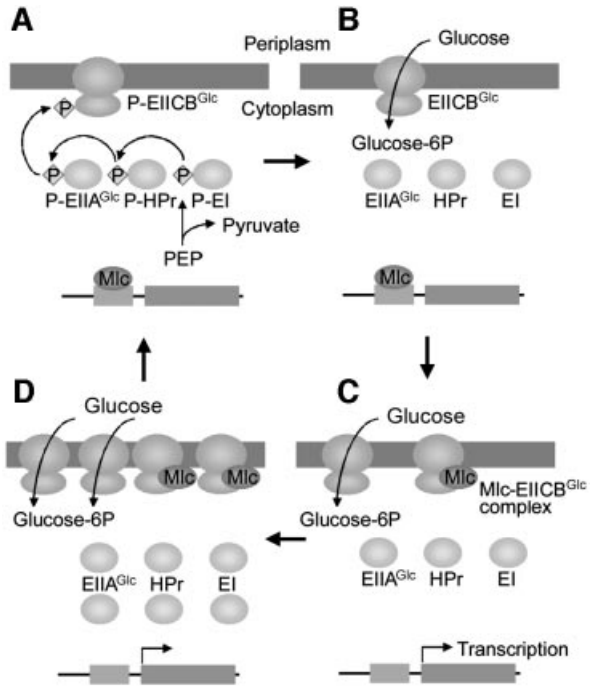

Fig. 6. A model showing that glucose induction is mediated by the direct interaction between Mlc and EIICB^{Glc} in a phosphorylation state-dependent manner. In the absence of glucose, PTS proteins are phosphorylated by PEP, and the phospho-form of EIICB^{Glc} does not interact with Mlc. The free Mlc binds to and represses its target genes, such as those for PTS proteins necessary for glucose transport (**A**). In the presence of glucose, PTS proteins are dephosphorylated, as glucose entering the bacteria is phosphorylated (**B**). The resulting unphosphorylated EIICB^{Glc} binds Mlc and sequesters it from binding to its target promoters, thus derepressing gene expression to make more PTS proteins necessary for the efficient uptake of glucose (**C**). As EIICB^{Glc} is dephosphorylated faster than it can be rephosphorylated by PEP in the presence of glucose, the induced EIICB^{Glc} sequesters more Mlc, forming a positive feedback loop (**D**). When glucose is depleted, the overproduced Mlc dissociates from phosphorylated EIICB^{Glc} and rapidly shuts down its target genes, forming a negative feedback loop (A).

and result in induction of the expression of Mlc-regulated genes. We showed that overproduction of EIICB^{Glc} resulted in induction of *ptsHIcrr* as well as *ptsG in vivo*, whereas a lack of EIICB^{Glc} caused reduction of their expression. It was recently reported that *ptsG–lacZ* expression was enhanced only when *ptsG* was expressed from its own promoter on a multicopy plasmid, but not when the *ptsG* promoter was replaced by the *lac* promoter (Plumbridge, 1999). On the basis of this result, Plumbridge suggested that induction of *ptsG–lacZ* expression might not result from overproduction of EIICB^{Glc} but from an operator titration effect displacing Mlc from its chromosomal locations; Plumbridge also suggested that EIICB^{Glc} might possibly interact with Mlc. To clarify the mechanism for induction of Mlc-regulated genes by EIICB^{Glc} overproduction, as shown in Figure 1 and in previous reports (De Reuse and Danchin, 1991; Plumbridge, 1999), direct interaction between Mlc and PTS proteins was measured using purified Mlc, EI, HPr, EIIA^{Glc} and EIIB^{Glc}. In this report, we demonstrated that glucose induction is mediated by the direct interaction between the membrane-bound glucose transporter EIICB^{Glc} and Mlc, in a manner dependent on the phosphorylation state of EIICB^{Glc} *in vitro*.

Every protein component of the *E.coli* PTS involved in glucose uptake has been shown to regulate the activity of other proteins by direct protein–protein interactions. The regulatory functions of the PTS depend on the phosphorylation state of its components. Unphosphorylated EI interacts with and regulates the autophosphorylation activity of CheA to trigger chemotaxis towards PTS carbohydrates (Lux *et al*., 1995). Unphosphorylated HPr interacts with and stimulates the activity of glycogen phosphorylase (Seok *et al*., 1997a). Unphosphorylated EIIA^{Glc} inhibits glycerol kinase and several sugar permeases by a mechanism termed inducer exclusion (Hurley *et al*., 1993; Postma *et al*., 1993). Phosphorylated EIIA^{Glc} stimulates cAMP synthesis (Peterkofsky *et al*., 1993). Here, we present the first evidence for the involvement of membrane-bound EIICB^{Glc} in the regulation of expression of several genes, by direct interaction with the global repressor Mlc in a phosphorylation state-dependent manner in *E.coli*; this is illustrated by the model in Figure 6. When glucose is phosphorylated in the course of transport, PTS proteins are dephosphorylated. The resulting increase in unphosphorylated EIICB^{Glc} causes the increase in the formation of the Mlc–EIICB^{Glc} complex, derepressing expression of its target genes. Mlc controls the *mlc* gene itself, as well as the *pts* operon coding for EI, HPr and EIIA^{Glc}, and the *ptsG* gene encoding EIICB^{Glc}. Since the *ptsG* promoter is much stronger than that of *mlc*, and the concentration of Mlc in *E.coli* is limiting (Kimata *et al*., 1998; Kim *et al*., 1999), the increased level of EIICB^{Glc} should completely sequester the induced level of Mlc. Thus, cells will make more PTS proteins necessary for the uptake of their sugar substrate, constituting a positive feedback loop. When glucose is depleted, the overproduced Mlc will rapidly dissociate from the phosphorylated EIICB^{Glc} and shut down its target genes, forming a negative feedback loop (Figure 6).

It was shown previously that the levels of EI and HPr in *E.coli* and *Salmonella typhimurium* were higher in cells grown in media containing glucose, fructose or mannitol than in cells grown in glycerol or lactate (Mattoo and Waygood, 1983). Since all PTS and several non-PTS sugars can also lower the phosphorylation state of the PTS proteins (Postma *et al*., 1993; Hogema *et al*., 1998), these sugars might also enhance the interaction between EIICB^{Glc} and Mlc to induce expression of the PTS proteins.

Materials and methods

Strains and plasmids

A *ΔptsG* mutant of *E.coli* strain GI698 (LaVallie *et al*., 1993) was constructed by P1 transduction of the Cm^R region from *E.coli* SR704, in which the *ptsG* gene is replaced by the chloramphenicol resistance gene, into *E.coli* GI698 (Seok *et al*., 1996), which encodes the gene for the λ c*I* repressor under control of the *trp* promoter. The DNA sequence from nucleotide 18 to 1505 of the *E.coli ptsG* gene (DDBJ/EMBL/GenBank accession No. J02618) was amplified by PCR, using mutagenic primers to create an *Nde*I site (underlined) at the ATG start codon (5′-TACTCAGGAGCACTCTCACATATGTTTAAG-3′) and a *Bam*HI site 15 nucleotides downstream from the TAA stop codon (5′-CTGGCTGCCTTAGGATCCCCAACGTCTTAC-3′). The PCR product digested with *Nde*I and *Bam*HI was cloned into vector pRE1 (Reddy *et al*., 1989), resulting in the recombinant plasmid pRE1-*ptsG* for EIICB^{Glc} overproduction. pRE1-*ptsHIcrr* was prepared by employing similar procedures to overproduce EI, HPr and EIIA^{Glc} simultaneously. The recombinant plasmid pJHK for overexpression of the EIIB domain of EIICB^{Glc} was prepared similarly, using a forward primer possessing the

synthetic *Nde*I site (underlined) at the new ATG start codon (5′-GCCGGGTCGTGAAGACCATATGGAAGATGC-3′) and the reverse primer used to make pRE1-*ptsG*. In pJHK, Thr390 of EIICB^{Glc} was changed into a new N-terminal Met to make the EIIB protein with 88 amino acids.

Protein purification

To purify Mlc in a day, we devised a single column chromatography procedure. In a previous report (Kim *et al*., 1999), we showed that overproduced Mlc was insoluble at neutral pH but could be solubilized in glycine–NaOH buffer pH 9.5. Thus, the cell pellet containing overexpressed Mlc was resuspended in 10 mM Tris–HCl pH 7.5 containing 50 mM NaCl, disrupted by passing twice through a French press at 10 000 p.s.i., and centrifuged at 10 000 *g* for 5 min to precipitate Mlc. The Mlc pellet was solubilized using 10 mM glycine–NaOH pH 9.5 containing 50 mM NaCl. After removing insoluble cell debris by centrifugation at 10 000 *g* for 5 min, the Mlc was ~90% pure. Solubilized Mlc was further chromatographed through an FPLC Mono Q 5/5 column (Pharmacia) using a gradient of 50–500 mM NaCl in 10 mM glycine–NaOH pH 9.5 (total volume 20 ml) to obtain homogeneous Mlc (>95% pure). *Escherichia coli* GI698 transformed with pRE1-*ptsHIcrr* was used for overproduction of the soluble PTS proteins (EI, HPr and EIIA^{Glc}), and these proteins were purified as described previously (Seok *et al*., 1996). To overexpress the EIIB domain of EIICB^{Glc}, *E.coli* GI698Δ*pts* (Nosworthy *et al*., 1998) transformed with pJHK was used. The EIIB domain was purified to homogeneity according to the procedure previously described for the purification of the N-terminal domain of *E.coli* EI (Seok *et al*., 1996).

Preparation of membrane vesicles

EIICB^{Glc}-enriched membrane vesicles were prepared from *E.coli* GI698Δ*ptsG* transformed with the pRE1-*ptsG* plasmid after induction by adding tryptophan to a final concentration of 100 μg/ml (Seok *et al*., 1997a), whereas membrane vesicles lacking EIICB^{Glc} were prepared from *E.coli* GI698Δ*ptsG* cells transformed with pRE1 according to the method described previously, with slight modifications (Seok *et al*., 1997b). The prepared membranes were washed twice with buffer containing 1 M NaCl and 2 mM dithiothreitol (DTT), and pelleted again at 100 000 *g* for 90 min. The resulting membrane vesicles were resuspended in 100 mM Tris–HCl pH 7.5 containing 2 mM DTT (25 mg protein/ml), and stored frozen at –80°C until use. Protein was estimated by using bicinchoninic acid protein assay reagents (Pierce). Expression of EIICB^{Glc} was estimated to constitute 15% of the total membrane protein.

Determination of the direct interaction between EIICB^{Glc} and Mlc

Incubation mixtures contained 100 mM Tris–HCl pH 7.5, 2 mM DTT, 2 mM $MgCl_2$, three soluble PTS proteins (5 μg of EI, 15 μg of HPr, 15 μg of EIIA^{Glc}) and 10 μg of Mlc, with the indicated amounts of membrane vesicles in a total volume of 100 μl. Incubations were carried out in polyallomer tubes (5 × 20 mm) designed for use in the Beckman Airfuge (Seok *et al*., 1997b). After incubation for 5 min at room temperature, the membrane vesicles with bound proteins were separated by centrifugation at 100 000 *g* for 15 min in an Airfuge. The pellets were resuspended in 100 μl of SDS–PAGE loading buffer, and 5 μl aliquots were run on SDS–PAGE gels along with 5 μl of each supernatant. To measure the binding affinity of the interaction, Mlc was incubated with various concentrations of membrane-bound EIICB^{Glc} with 2 mM glucose in the presence of EI, HPr and EIIA^{Glc}. After centrifugation, the supernatants containing unbound Mlc were examined by SDS–PAGE using a 15% polyacrylamide gel. After the gel was stained with Coomassie Blue, the amounts of unbound Mlc were quantified by densitometric tracing of the stained gel. The dissociation constant (K_d) was calculated by plotting the amount of bound Mlc, obtained by subtracting unbound from added Mlc, versus added EIICB^{Glc}.

Phosphorylation state-dependent interaction between EIIB and Mlc

Real-time interactions of Mlc with the EIIB domain and other soluble PTS proteins were monitored by SPR detection under different conditions using a BIAcore 2000 (Pharmacia Biosensor AB, Uppsala, Sweden). EI, HPr and EIIA^{Glc} were separately immobilized on a CM5 sensor chip as described previously (Seok *et al*., 1997a). The EIIB domain (60 μl, 20 μg/ml) in coupling buffer (10 mM Na acetate pH 5.0) was allowed to flow over a CM5 sensor chip at 10 μl/min to couple the protein to the carboxymethylated dextran matrix by a NHS/EDC reaction (70 μl of mix). Unreacted *N*-hydroxysuccinimide was inactivated by injecting 70 μl of 1 M ethanolamine–HCl pH 8.0. The EIIB domain was immobilized to a surface concentration of 1.2 ng/mm^2. The standard running buffer was 10 mM HEPES pH 7.2, 150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA and 1 mM DTT, and all reagents were introduced at a flow rate of 10 μl/min. To phosphorylate the immobilized EIIB domain, a mixture (50 μl) of PEP (0.1 mM) and three soluble PTS proteins (10 μg/ml each) in the standard running buffer was allowed to flow into the flow cell for 5 min. To remove the phosphoryl group from the immobilized phospho-EIIB domain, EIIA^{Glc} (0.1 mg/ml in the standard running buffer) was allowed to flow in the flow cell for 5 min. The sensor surface was regenerated between injections by flowing the standard running buffer at a flow rate of 100 μl/min for 10 min to remove bound analytes.

Gel shift assay

DNA fragments covering the promoter regions of *ptsG*, *pts* P0 and *mlc* (from –264 to +108, –249 to +211 and –146 to +183 with respect to the transcriptional start sites, respectively) were amplified by PCR and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The binding buffer for the electrophoretic mobility shift assays contained 100 mM HEPES pH 8.0, 25 mM monosodium glutamate and 1 mg/ml bovine serum albumin, in a total volume of 10 μl. The binding mixtures were incubated at room temperature for 10 min, and analyzed by electrophoresis on 6% polyacrylamide gels in 0.5 × TBE at room temperature for 90 min.

In vivo transcript analysis

For S1 nuclease protection analysis, a DNA fragment covering the *ptsG* promoter region (from –363 to –1 with respect to the translational start codon) was amplified by PCR and used as the probe to detect mRNA originating from chromosomal *ptsG* but not from the plasmid pRE1-*ptsG*. RNA was prepared from GI698 cells harboring pRE1, pRE1-*ptsG* or pRE1-*ptsHIcrr* grown in synthetic medium containing 0.5% glycerol as carbon source (Seok *et al*., 1996). Cells were grown to A_{600} = 1.0 with or without induction at A_{600} = 0.5 by adding 100 μg/ml of tryptophan. For each sample, 150 μg of RNA were hybridized with 60 000 c.p.m. of the ^{32}P-labeled probe, followed by S1 nuclease digestion as described previously (Smith, 1991). The protected fragments were electrophoresed on a 6% polyacrylamide gel containing 7 M urea. Primer extension analyses were carried out as described previously (Kim *et al*., 1999) using RNA samples (30 μg each) prepared from the indicated strains grown in tryptone broth (Kim *et al*., 1999) with or without glucose.

In vitro transcription assay

Reactions were carried out as described previously in a total volume of 25 μl, with slight modifications (Ryu and Garges, 1994; Kim *et al*., 1999). Mlc (100 ng), EI (200 ng), HPr (200 ng), EIIA^{Glc} (200 ng) and membrane vesicles (500 ng of protein) were added as indicated. Membrane-bound EIICB^{Glc} was phosphorylated by adding 2 mM PEP, and dephosphorylated by adding 2 mM glucose to the transcription buffer in the presence of EI, HPr and EIIA^{Glc}, and incubating at room temperature for 5 min. The transcription mixture was incubated at 37°C for 10 min before adding nucleotides. Transcription was initiated by adding nucleotides and terminated after 10 min by adding 25 μl of formamide loading buffer. RNA was resolved by electrophoresis using 6% polyacrylamide gels with 8 M urea.

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Two papers were recently published presenting data documenting the interaction between $EIICB^{Glc}$ and Mlc: Lee,S.J., Boos,W., Bouche,J.P. and Plumbridge,J. (2000) Signal transduction between a membrane-bound transporter, PtsG, and a soluble transcription factor, Mlc, of *Escherichia coli. EMBO J*., **19**, 5353–5361. Tanaka,Y., Kimata,K. and Aiba,H. (2000) A novel regulatory role of glucose transporter of *Escherichia coli*: membrane sequestration of a global repressor Mlc. *EMBO J*., **19**, 5344–5352.