BglF, the Sensor of the bgl System and the β-Glucosides Permease of Escherichia coli : Evidence for Dimerization and Intersubunit Phosphotransfer †

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BglF, the Sensor of the *bgl* System and the β-Glucosides Permease of *Escherichia coli*: Evidence for Dimerization and Intersubunit Phosphotransfer†

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ABSTRACT: The *Escherichia coli* BglF protein, also designated EII^{bgl}, is an enzyme II of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) that catalyzes transport and phosphorylation of β-glucosides. In addition, BglF has the ability, unusual for an EII, to regulate the activity of a transcriptional regulator, BglG, by phosphorylating and dephosphorylating it according to β-glucoside availability. Together, BglF and BglG constitute a novel sensory system. The membrane-bound sensor, BglF, has two phosphorylation sites: site 1 accepts a phosphoryl group from HPr and delivers it to site 2; site 2 delivers the phosphoryl group either to β-glucosides or to BglG. Here, we provide several lines of evidence for the dimerization of BglF and for the occurrence of productive intersubunit phosphotransfer within the BglF dimers. (1) Two inactive BglF mutant proteins, one lacking phosphorylation site 1 and the other lacking site 2, complement one another to allow β-glucoside utilization by *bglF* strains. (2) The pairs of mutant proteins complement one another in regulating BglG activity as a transcriptional antiterminator in vivo. (3) Only when they are present in the same membrane preparation do the mutant protein pairs efficiently transfer the phosphoryl group from HPr to β-glucosides and to BglG in vitro. (4) Gentle extraction of cellular proteins followed by SDS−PAGE reveals the existence of BglF homodimers. A portion of the phosphorylated form of BglF can also be extracted from the membrane as a dimer. Dimerization is mediated by the membrane-bound IIC^{bgl} domain, as indicated by the dimerization of IIC^{bgl} by itself and of BglF derivatives that contain this domain. Since dimers persist in the presence of a reducing agent, they are apparently not held together by disulfide bonds. Rather, BglF dimerization might involve hydrophobic interactions between residues in the membrane-spanning domain. In addition, we show that BglF dimerization is not modulated by β-glucosides and is therefore not part of the mechanism that diverts the phosphoryl group away from BglG to the transported sugar upon addition of β-glucosides to the growth medium.

The *Escherichia coli* BglF protein, a member of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), catalyzes transport and concomitant phosphorylation of β-glucosides (*1*). The phosphoryl flux in PTS starts with a phosphoryl group, donated by PEP, which is passed through the general PTS proteins, enzyme I (EI) and HPr, to the various sugar-specific enzymes II (EIIs). Like many other EIIs, BglF (EII^{bgl}) consists of three well-defined domains: IIA^{bgl} possesses the first phosphorylation site, His547, which can be phosphorylated by P−HPr; IIB^{bgl} possesses the second phosphorylation site, Cys24, which accepts the phosphoryl group from P−IIA^{bgl} and transfers it to β-glucosides; IIC^{bgl}, the membrane-spanning domain, presumably forms the sugar translocation channel and at least part of the sugar binding site (*2*−*4*). The order of these domains in the BglF polypeptide is BCA, and thus, it can be designated IIBCA^{bgl}.

In addition to its role in sugar phosphotransfer, BglF negatively regulates expression of the *bgl* operon (*5*). This property of BglF relies on its unprecedented ability to phosphorylate not only its sugar substrate but also a regulatory protein, BglG (*6*−*8*). BglG is an RNA-binding protein which controls *bgl* operon expression by transcription antitermination (*9*−*11*). Together, BglF and BglG constitute a novel sensory system which responds to the presence of β-glucosides in the environment by inducing expression of the *bgl* operon (*12*, *13*). By reversibly phosphorylating BglG, BglF modulates the dimeric state of BglG and thus regulates its activity (*14*). Interestingly, the same phosphorylation site on BglF, Cys24, transfers phosphoryl groups to both substrates, the sugar and the BglG protein (*4*). The mechanism that ensures correct delivery of the phosphoryl group to the right entity, sugar or protein, by the same active site is not known.

Arguments that are based on the number of EII molecules which are required for forming a large enough channel to admit a sugar suggest that an oligomer might be necessary for the formation of the channel (*3*, *15*). Indeed, the

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[1] Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; PEP, phosphoenolpyruvate; HPr, histidine-containing phosphocarrier protein; EII^{bgl}, β-glucoside-specific enzyme II; EII^{glc}, glucose-specific enzyme II; EII^{mtl}, mannitol-specific enzyme II; EII^{man}, mannose-specific enzyme II; EII^{nag}, *N*-acetylglucosamine-specific enzyme II; ATP, adenosine 5′-triphosphate; cAMP, adenosine 3′:5′-cyclic monophosphate; MBP, maltose-binding protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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Table 1: Plasmids Encoding BglF Derivatives

plasmid	encoded BglF derivative(s)	reference
pT7OAC-F	wild-type BglF (IIBCA^{bgl})	*6*
pT7CQ-F1	H547R	*4*
pT7CQ-F2	C24S	*4*
pT7CQ-F3	IIB^{bgl}	this work
pT7CQ-F4	IIC^{bgl}	this work
pT7CQ-F5	IIA^{bgl}	this work
pT7CQ-F6	IICA^{bgl}	this work
pT7CQ-F8	IIBC^{bgl}	this work
pT7CQ-F9	BglF-like (IIBCA^{bgl} with longer linkers between the domains)	this work
pT7CQ-F11	C24S and IIBC^{bgl}	this work
pT7CQ-F12	H547R and IICA^{bgl}	this work
pMN5	wild-type BglF (IIBCA^{bgl})	*5*
pCQ-F1	H547R	*4*
pCQ-F2	C24S	*4*
pCQ-F11	C24S and IIBC^{bgl}	this work
pCQ-F12	H547R and IICA^{bgl}	this work

existence of dimeric EII^{mtl}, the mannitol permease, has been demonstrated directly by various methods (*16*−*21*). Productive intersubunit phosphotransfer between the subunits of the EII^{mtl} dimer was observed both in vitro and in vivo (*22*−*25*). The evidence strongly suggests that dimeric EII^{mtl} is the predominant and more active species under physiological conditions. Dimerization of EII^{man} has also been demonstrated (reviewed in ref *3*).

In this paper, we provide indirect and direct evidence for BglF dimerization. We show that two BglF mutant proteins, each lacking one phosphorylation site, can complement one another and implement β-glucoside phosphotransfer and BglG negative regulation in vivo when they are coexpressed in the cell. Productive phosphotransfer between these mutant proteins that allows them to transfer the phosphoryl group from P−HPr to the sugar or to BglG in vitro occurs, provided that the two proteins are present in the same membrane preparation. These results strongly suggest that functional heterodimers are formed between the complementary mutant proteins. Direct evidence for the existence of BglF dimers, as well as P−BglF dimers, was obtained by analyzing wild-type and mutant BglF proteins by SDS−PAGE after gentle extraction from the membrane. Dimerization of the IIC^{bgl} domain by itself, and of truncated BglF proteins that contain this domain, was demonstrated. This finding, together with the insensitivity of the dimeric form of BglF to reducing agents, suggests that the dimers might be held together by hydrophobic interactions between residues in the IIC^{bgl} domain. No change in the oligomeric state of BglF was observed upon addition of β-glucoside. Therefore, the ability of BglF to form dimers that can carry out sugar-stimulated and nonstimulated phosphorylation functions seems to be an inherent property of the protein.

EXPERIMENTAL PROCEDURES

Strains. The following *E. coli* K12 strains were used. K38 (HfrC *trpR thi* λ^+) was obtained from C. Richardson. LM1 contains mutations in the *nagE* and *crr* genes, which code for EII^{nag} and IIA^{glc}, respectively (*26*). PPA543 and PPA547 contain mutations in the *crr*, *nagE*, and *bglF* genes; the latter also carries a *bgl′*−*lacZ* fusion on its chromosome (*4*).

Plasmids. The plasmids that encode BglF derivatives are listed in Table 1. Plasmids pT712 and pT713, which contain the phage T7 late promoter, and plasmid pGP1-2, which carries the T7 RNA polymerase gene under control of the λ CI857 repressor, were obtained from Bethesda Research Laboratories. Plasmid pT7FH-G carries the entire *bglG* gene cloned downstream of the T7 promoter in pT713; plasmid pT7OAC-F carries the entire *bglF* gene cloned downstream of the T7 promoter in pT712 (*6*). Plasmids pT7CQ-F1 and pT7CQ-F2 are derivatives of pT7OAC-F that encode BglF with either His547 mutated to Arg (H547R) or Cys24 mutated to Ser (C24S), respectively (*4*). Plasmid pMN5 carries the entire *bglF* gene cloned into pBR322 (*5*). Plasmids pCQ-F1 and pCQ-F2 are derivatives of pBR322 that encode BglF which contains either the H547R or C24S mutation, respectively (*4*). Additional plasmids were constructed as described below.

Plasmid pTQ contains a 371 bp *Eco*RI−*Pvu*II fragment from pQE-30, which contains the sequence encoding six histidines and a translation start site followed by a multicloning site, ligated to the 2592 bp *Eco*RI−*Pvu*II fragment of pT713, which contains the T7 promoter.

Plasmid pTQD is a derivative of PTQ which is deleted for the sequence encoding the six histidines. It was constructed by overlap extension with PCR (*27*).

Plasmid pT7CQ-F3 encodes IIB^{bgl} cloned downstream of the T7 promoter in pTQD. A 233 bp fragment, encoding IIB^{bgl} and containing a *Bam*HI site at one end and a *Kpn*I site at the other, was generated by PCR, using pT7OAC-F as a template. This fragment was ligated to the 2945 bp *Bam*HI−*Kpn*I fragment of pTQD.

Plasmid pT7CQ-F4 encodes IIC^{bgl} cloned downstream of the T7 promoter in pTQD. A 1148 bp *Stu*I−*Bsp*1286I fragment from pT7OAC-F, encoding IIC^{bgl}, was purified. After the *Bsp*1286I site was converted to a *Pst*I site by using a synthetic linker, this fragment was ligated to the 2945 bp *Sma*I−*Pst*I fragment of pTQD.

Plasmid pT7CQ-F5 encodes IIA^{bgl} cloned downstream of the T7 promoter in pTDQ. A 572 bp *Bsp*1286I−*Sma*I fragment from pT7OAC-F, encoding IIA^{bgl}, was purified, and the *Bsp*1286I site was converted to a *Pst*I site by using a synthetic linker. The resulting fragment was ligated to a 2815 bp fragment from pTQD prepared by digesting pTQD DNA with *Nhe*I, filling in the 5′ overhangs with Klenow fragment, and cleaving with *Pst*I.

Plasmid pT7CQ-F6, which encodes IICA^{bgl} cloned downstream of the T7 promoter, was constructed by ligating a 844 bp *Pst*I−*Pvu*II fragment from pT7CQ-F5 (encoding IIA^{bgl}) to a 3813 bp *Pst*I−*Pvu*II fragment from pT7CQ-F4 (encoding IIC^{bgl}).

Plasmid pT7CQ-F8, which encodes IIBC^{bgl} cloned downstream of the T7 promoter, was constructed by ligating a 1159 bp *Kpn*I−*Pst*I fragment from pT7CQ-F4 (encoding IIC^{bgl}) to a 3163 bp *Kpn*I−*Pst*I fragment from pT7CQ-F3 (encoding IIB^{bgl}).

Plasmid pT7CQ-F9, which encodes IIBCA^{bgl} (BglF with longer linkers between the domains, designated BglF-like) cloned downstream of the T7 promoter, was constructed by ligating a 714 bp *Pst*I−*Pvu*II fragment from pT7CQ-F5 (encoding IIA^{bgl}) to a 4042 bp *Pst*I−*Pvu*II fragment from pT7CQ-F8 (encoding IIBC^{bgl}).

Plasmid pT7CQ-F11, which encodes both the C24S BglF mutant protein and IIBC^{bgl} cloned downstream of the T7 promoter, was constructed by ligating a 1730 bp *Eco*RI−

*Pvu*II fragment from pT7CQ-F8 (encoding $IIBC^{bgl}$) to a 4709 bp *Eco*RI−*Pvu*II fragment from pT7CQ-F2 (encoding the C24S mutant protein).

Plasmid pT7CQ-F12, which encodes both the H547R BglF mutant protein and $IICA^{bgl}$ cloned downstream of the T7 promoter, was constructed by ligating a 1950 bp *Eco*RI−*Pvu*II fragment from pT7CQ-F6 (encoding $IICA^{bgl}$) to a 4709 bp *Eco*RI−*Pvu*II fragment from pT7CQ-F1 (encoding the H547R mutant protein).

Plasmid pCQ-F11, a pBR322 derivative which encodes both the C24S BglF mutant protein and $IIBC^{bgl}$, was constructed by ligating a 1730 bp *Eco*RI−*Pvu*II fragment from pT7CQ-F8 (encoding $IIBC^{bgl}$) to a 6301 bp *Eco*RI−*Ssp*I fragment from pCQ-F2 (encoding the C24S mutant protein).

Plasmid pCQ-F12, a pBR322 derivative which encodes both the H547R mutant and $IICA^{bgl}$, was constructed by ligating a 2065 bp *Eco*RI−*Pvu*II fragment from pT7CQ-F6 (encoding $IICA^{bgl}$) to a 6301 bp *Eco*RI−*Ssp*I fragment from pCQ-F1 (encoding the H547R mutant protein).

Chemicals. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Rotem Industries Ltd. (Israel). [^{35}S]Methionine (1200 Ci/mmol) was obtained from DuPont. PEP, pyruvic acid, and pyruvate kinase were obtained from Sigma. [^{32}P]PEP was prepared and separated from [^{32}P]ATP as described previously (*6*). Purified enzyme I and HPr were obtained from J. Reizer. MBP−BglG was purified as described previously (*4*).

Media. Enriched media, M9 salts, and M63 salts minimal media were prepared essentially as described by Miller (*28*). The minimal medium used for [^{35}S]methionine labeling was the same as that used by Tabor and Richardson (*29*), with 0.4% succinate added as a carbon source. Ampicillin (200 μg/mL) or kanamycin (30 μg/mL) was included in the media when growing strains which contain plasmids that confer resistance to either one of these antibiotics. MacConkey arbutin plates were prepared as described previously (*30*).

Molecular Cloning. All manipulations with recombinant DNA were carried out by standard procedures (*31*). Restriction enzymes and other enzymes used in recombinant DNA experiments were purchased commercially and were used according to the specifications of the manufacturers.

Measurements of β-Galactosidase Activity. Assays for β-galactosidase activity were carried out as described by Miller (*28*). Cells were grown in minimal medium which was supplied with 0.2% lactate as a carbon source. Because the strains used carried a *crr* mutation, we supplemented the medium with 5 mM cAMP.

Preparation of Membrane Fractions. Membrane fractions enriched for the various BglF proteins were prepared as described before (*6*). The proteins were expressed from their respective genes under T7 promoter control in plasmids pT7OAC-F, pT7CQ-F1, pT7CQ-F2, pT7CQ-F6, pT7CQ-F8, pT7CQ-F9, pT7CQ-F11, and pT7CQ-F12. Expression of T7 RNA polymerase from plasmid pGP1-2, which is compatible with the pT7 plasmids, was induced thermally. The *E. coli* strain LM1 was used as a host.

Membrane fractions lacking BglF were prepared from strain LM1/pGP1-2/pT712 and were used in control experiments.

In Vitro Phosphorylation. In vitro phosphorylation was carried out essentially as described by Chen et al. (*4*). Briefly, membrane fractions with a final protein concentration of 0.9 mg/mL were labeled by incubation at 30 °C in a mixture containing 10 μg/mL enzyme I, 40 μg/mL HPr, 10 μM [^{32}P]PEP, and PLB buffer [50 mM Na_2HPO_4 (pH 7.4), 0.5 mM $MgCl_2$, 1 mM NaF, and 2 mM dithiothreitol]. Where indicated, two membrane fractions were added together to the mixture in equal amounts. After incubation for 10 min, reactions were either terminated by addition of electrophoresis sample buffer or incubated further as described below. To study dephosphorylation by β-glucosides, salicin was added to a final concentration of 0.2%, and incubation was continued at 30 °C for 5 min. To study BglG phosphorylation, purified MBP−BglG, which was first adjusted to the PLB buffer concentration indicated above, was added to a final concentration of 10 mM, and incubation was continued at 30 °C for 15 min.

Overproduction and [^{35}S]Methionine Labeling of BglF. To overproduce BglF or its derivatives, the different *bglF* alleles were cloned under T7 promoter control, as described above. The resulting plasmids were introduced into K38 cells containing pGP1-2, which encodes the T7 polymerase. Thermal induction and labeling with [^{35}S]methionine were carried out in the presence of rifampicin (Sigma), as described by Tabor and Richardson (*29*).

Electrophoresis and Autoradiography. Cells prelabeled with [^{35}S]methionine or mixtures of ^{32}P-labeled proteins were incubated in electrophoresis sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue, as follows. ^{32}P-labeled proteins were incubated for 30 min at 30 °C; ^{35}S-labeled proteins were incubated either for 5 min at 100 °C or for 30 min at 30 °C, as indicated. Electrophoresis of proteins was carried out on 10 or 5 to 12.5% (gradient) SDS−polyacrylamide gels as described by Laemmli (*32*). Alternatively, tricine−SDS−polyacrylamide gels with a 4% stacking gel and an 8 to 20% separating gel, prepared as described by Schagger and von Jagow (*33*), were used. Samples were fractionated next to prestained low- or midrange protein molecular mass markers (Amersham). After electrophoresis, gels were dried and exposed to Kodak XAR-5 X-ray film at −70 °C for periods varying from hours to weeks, depending on the isotope and the experiment. Molecular masses of radioactively labeled proteins were estimated using the Molecular program of the BAS1000 Bio-imaging analyzer.

Gel Purification of Suspected Dimers of BglF. ^{35}S-labeled BglF dimers were extracted from gels as follows. After fractionation of ^{35}S-labeled cellular proteins on a 5 to 12.5% SDS−polyacrylamide gel, as described above, the wet gel was exposed to X-ray film at 4 °C. A gel slice containing the labeled band which corresponds to the predicted size of dimers of BglF was excised, and the protein was extracted from it by incubation in electrophoresis sample buffer for 2 h at 30 °C. The gel-extracted protein was divided into two samples. One was incubated for 5 min at 100 °C, and the other was left at 30 °C. Both samples were analyzed on a 5 to 12.5% SDS−polyacrylamide gel.

RESULTS

Intersubunit Phosphotransfer in BglF Occurs in Vitro. Phosphotransfer in BglF occurs between His547, located in

the IIA^{bgl} domain (site 1), and Cys24, located in the IIB^{bgl} domain (site 2). The question arises as to whether the phosphoryl group is transferred within the same polypeptide, i.e., intramolecularly, or between two BglF molecules, i.e., intermolecularly. To determine if intersubunit phosphotransfer can occur in BglF, we examined the ability of two BglF derivatives, each inactive due to the lack of one phosphorylation site, to be phosphorylated and to implement the various BglF phosphorylation functions in vitro. The following pairs of mutants were examined: (1) BglF mutated in site 1, designated H547R, and a truncated BglF derivative which lacks the IIB domain, and thus lacks site 2, designated IICA^{bgl}; and (2) BglF mutated in site 2, designated C24S, and a truncated BglF derivative which lacks the IIA domain, and thus lacks site 1, designated IIBC^{bgl}. The composition of each pair from a full size and a truncated BglF proteins allowed the two components of the pair to be separated by SDS−PAGE.

Two parallel sets of in vitro phosphorylation experiments were performed. In one set, the proteins of each pair were coexpressed in the *E. coli* strain LM1, from which the *crr* and *nagE* genes have been deleted (and therefore does not express the IIA^{glc} and II^{nag} proteins, which can substitute for IIA^{bgl} and complement a mutation in site 1 of BglF), and membranes were prepared from these cells. In a complementary set of experiments, each of the pair components was expressed individually in strain LM1, and the two membrane preparations were subsequently mixed. The membranes were then incubated with [^{32}P]PEP and purified enzyme I and HPr.

We have shown previously that the C24S protein is phosphorylated in this in vitro system, whereas the H547R protein is not (*4*). The results presented in Figure 1 demonstrate that phosphorylation of H547R in this system occurrs when IICA^{bgl} is present in the same membrane preparation with H547R [Figure 1, lane 5 (H547R&CA coex.)]. However, when membranes containing H547R were mixed with membranes containing IICA^{bgl}, phosphorylated H547R could hardly be detected [Figure 1, lane 7 (H547R + CA)]. Therefore, IICA^{bgl} can efficiently transfer the phosphoryl group from HPr to H547R only when it is present in the same membrane as H547R. This result indicates that phosphotransfer from IICA^{bgl} to H547R occurs, provided that the two proteins are in physical proximity and most probably forming an oligomer in the membrane.

C24S, as expected, behaved like wild-type BglF, and its phosphorylation in this system did not depend on the presence of IIBC^{bgl} in the same membrane preparation, because C24S contains an intact site 1, which can accept the phosphoryl group from HPr [compare lanes 3 (wild-type BglF), 9 (C24S&BC coex.), and 11 (C24S + BC)]. Nevertheless, we could test indirectly whether the phosphoryl group can be transferred from site 1 in C24S to site 2 in IIBC^{bgl}. C24S can accept the phosphoryl group from HPr but cannot transfer it to the two substrates of BglF, β-glucosides and BglG, due to the mutation in site 2 (*4*), whereas IIBC^{bgl} cannot be phosphorylated by HPr but, once phosphorylated, can transfer the phosphoryl group to the BglF substrates. We tested whether this pair of proteins can work together to transfer the phosphoryl group from HPr to the β-glucoside salicin and to BglG. As seen in Figure 1, when phosphorylated C24S was present in the same membrane as IIBC^{bgl}, it behaved like the phosphorylated wild-type protein in its ability to be dephosphorylated upon the addition of salicin [Figure 1, compare lanes 3 and 4 (WT BglF) with lanes 9 and 10 (C24S&BC coex.)]. Dephosphorylation by salicin was much less efficient when C24S and IIBC^{bgl} were expressed individually in vivo and mixed in vitro [Figure 1, lanes 11 and 12 (C24S + BC)]. The same result was obtained even after prolonged periods of incubation with the sugar (data not shown). At least part of the reduction in intensity of phosphorylated C24S upon salicin addition (see lane 12 of Figure 1) can be attributed to the presence of IICB^{glc} in our membrane preparations. This protein can be phosphorylated by site 1 of the C24S protein and thereby drain phosphoryl groups from BglF because commercial salicin contains residual glucose contamination (*4*).

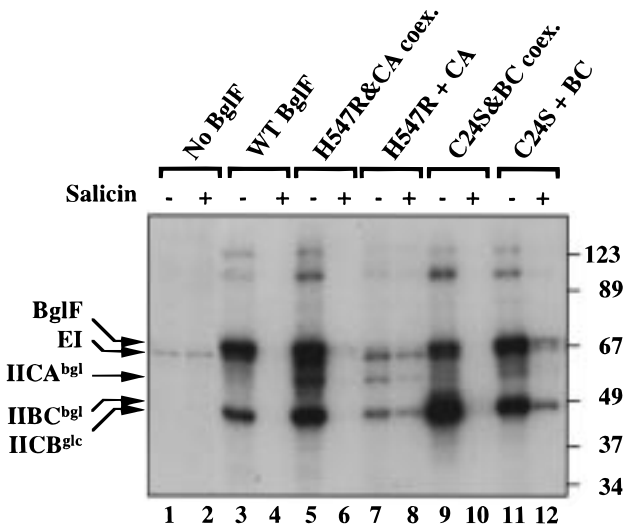

FIGURE 1: β-Glucoside phosphorylation is catalyzed by pairs of BglF mutant proteins, each lacking one phosphorylation site. The various BglF derivatives were overproduced in *E. coli* LM1 cells (see Experimental Procedures). Membrane fractions were incubated with [^{32}P]PEP and purified enzyme I and HPr for 10 min. The mixtures were further incubated with (+) or without (−) 0.2% salicin for 5 min. Samples were analyzed on 10% SDS−polyacrylamide gels followed by autoradiography. Molecular masses of protein standards are given in kilodaltons. Arrowheads indicate the position of BglF, enzyme I (EI), IICA^{bgl}, IIBC^{bgl}, and IICB^{glc}. WT BglF = wild-type BglF. H547R&CA coex. = H547R and IICA^{bgl} coexpressed. H547R + CA = H547R and IICA^{bgl} expressed individually and the membrane fractions subsequently mixed. C24S&BC coex. = C24S and IIBC^{bgl} coexpressed. C24S + BC = C24S and IIBC^{bgl} expressed individually and the membrane fractions subsequently mixed.

As for the other pair, efficient dephosphorylation of H547R by salicin was observed when this protein was present in the same membrane with IICA^{bgl} [Figure 1, lanes 5 and 6 (H547R&CA coex.)]. Dephosphorylation of H547R expressed individually and subsequently mixed with IICA^{bgl} could not be studied due to the inability of H547R to be phosphorylated under these conditions [Figure 1, lanes 7 and 8 (H547R + CA)].

The results presented thus far show that the phosphoryl group is transferred from site 1 of one BglF molecule to site 2 of another molecule and that the transfer is much more efficient when the two molecules are present in the same membrane. These data provide a strong indication that BglF forms an oligomer in the membrane, most probably a dimer, that functions in β-glucoside phosphorylation. However,

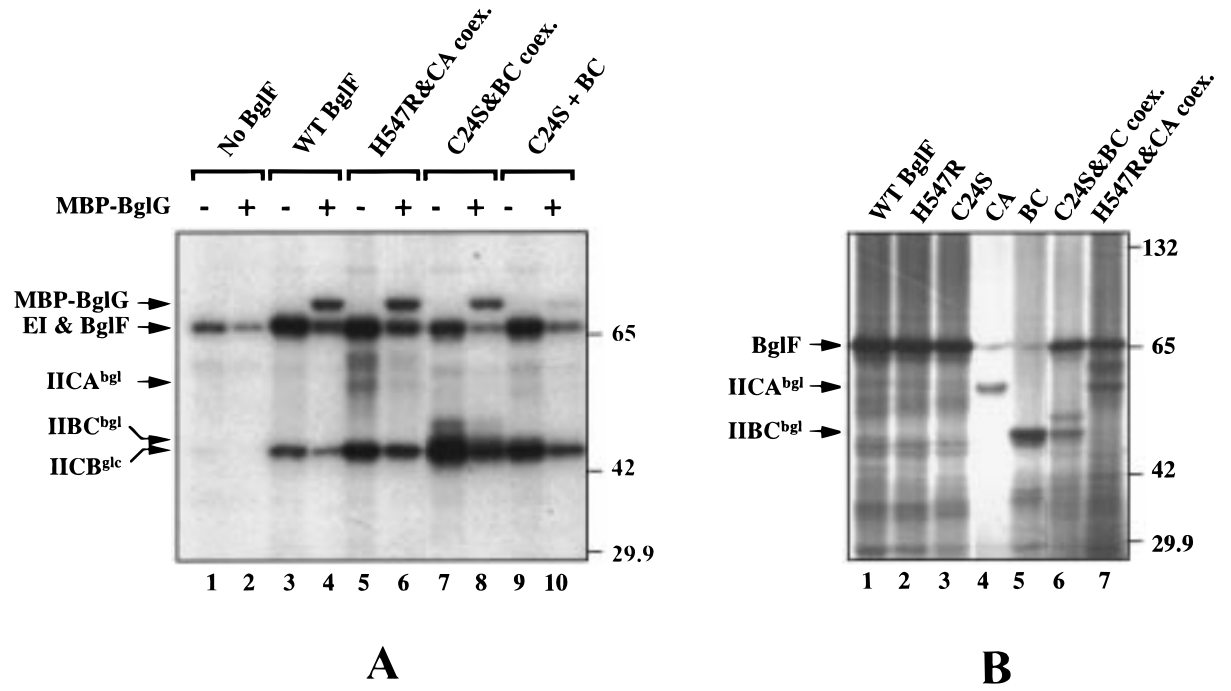

FIGURE 2: BglG phosphorylation is catalyzed by pairs of BglF mutant proteins, each lacking one phosphorylation site. (A) Membranes containing the various BglF derivatives were incubated with [^{32}P]PEP and purified enzyme I and HPr for 10 min. The mixtures were further incubated in the presence (+) or absence (−) of MBP−BglG for 15 min. Proteins were fractionated on 5 to 12.5% gradient SDS−polyacrylamide gels followed by autoradiography. The first two lanes contain a control with membranes from cells that do not overproduce BglF. Arrowheads indicate the positions of MBP−BglG, BglF, and enzyme I (EI). BglF and EI comigrate in this gel system. (B) Overproduction of BglF and its derivatives was induced in *E. coli* K38 cells, and the cellular proteins were labeled with [^{35}S]methionine for 2 min (see Experimental Procedures). Proteins were extracted at 100 °C for 5 min in electrophoresis sample buffer, and equal volumes were analyzed on a 10% SDS−polyacrylamide gel followed by autoradiography. Arrowheads indicate the positions of BglF and its truncated forms IICA^{bgl} and IIBC^{bgl}. Molecular masses of protein standards are given in kilodaltons. WT BglF = wild-type BglF. H547R&CA coex. = H547R and IICA^{bgl} coexpressed. C24S&BC coex. = C24S and IIBC^{bgl} coexpressed. C24S + BC = C24S and IIBC^{bgl} expressed individually and the membrane fractions subsequently mixed.

they do not address whether this form functions in BglG phosphorylation. To test whether the described pairs of BglF mutant proteins can act in concert to phosphorylate BglG, we assayed the ability of the prelabeled pairs to donate the phosphoryl group to BglG. BglG fused to maltose-binding protein (MBP−BglG) was used as a substrate, since the hybrid protein can be readily purified on an amylose column. We have previously shown that the BglG moiety of MBP−BglG is recognized and phosphorylated by BglF in vitro (*4*).

The results presented in Figure 2A demonstrate that each pair, when its components were coexpressed in vivo, behaved like wild-type BglF in its ability to deliver the phosphoryl group to BglG in vitro [Figure 2A, compare lanes 3 and 4 (WT BglF) with lanes 5 and 6 (H547R&CA coex.) and lanes 7 and 8 (C24S&BC coex.)]. In contrast, BglG was poorly phosphorylated when C24S and IIBC^{bgl} were expressed individually in vivo and mixed in vitro [Figure 2A, lanes 9 and 10 (C24S + BC)]. Phosphorylation of BglG by H547R and IICA^{bgl} expressed individually is not expected to occur, because phosphotransfer between these mutants occurs only when they are coexpressed (see Figure 1, lane 7). Indeed, when MBP−BglG was added to these proteins when they were expressed individually and then preincubated together in the phosphorylation system, phosphorylated MBP−BglG could not be detected (data not shown). In a control experiment, MBP−BglG was added to membranes from cells that do not produce BglF, which were preincubated with [^{32}P]-PEP and purified enzyme I and HPr (Figure 2A, lanes 1 and 2). No phosphorylated MBP−BglG was detected. Thus, consistent with our previously published results (*4*), BglG cannot be phosphorylated directly by enzyme I and HPr.

To verify that the better performance of the coexpressed pairs versus the individually expressed BglF mutants was not due to higher levels of the coexpressed proteins, we compared the levels of the respective proteins in the cell. To this end, we labeled the various mutants in vivo with [^{35}S]methionine and compared their intensities by autoradiography after SDS−PAGE. The results, presented in Figure 2B, demonstrate that the level of each of the BglF derivatives (H547R, C24S, IICA^{bgl}, and IIBC^{bgl}) was higher when it was expressed individually (Figure 2B, lanes 2−5, respectively) than when coexpressed (lanes 6−7). In addition, all the mutants, expressed together or alone, were stable during a 60 min chase with unlabeled methionine (data not shown). Therefore, the ability of the coexpressed mutant protein pairs to carry out the BglF phosphorylation functions is not due to them being synthesized at higher levels than the individually expressed mutants.

Taken together, these results demonstrate a cooperative action of two BglF molecules in transferring the phosphoryl group from HPr to both BglF substrates, β-glucosides and BglG, in vitro. The requirement that the two molecules be in the same membrane suggests that they interact via their membrane-embedded portions. This conclusion is supported by the apparent dependence of this interaction on the membrane-bound IIC^{bgl} domain, which is deduced from the fact that this domain is the only one which is shared by all the complementing components.

Table 2: β-Glucoside Phosphotransfer and BglG Negative Regulation Can Be Implemented in Vivo by Pairs of BglF Mutant Proteins, Each Lacking One Phosphorylation Site

Plasmid	Plasmid-encoded BglF derivative	BglF domains	Complementation[a] of *bglF* mutant strains[b]	β-gal activity (U)[c]	
				- Salicin	+ Salicin
pCQ-F	Wild type	● C24 — ● H547	+	4	126
pCQ-F1	H547R	● — ✕ H547R	-	179	340
pCQ-F2	C24S	✕ C24S — ●	-	70	138
pCQ-F11	C24S & IIBC^{bgl}	✕ C24S — ●; ●	+	5	140
pCQ-F12	H547R & IICA^{bgl}	● — ✕ H547R; ●	+	5	189

[a] Complementation was indicated by growth of red colonies on MacConkey arbutin plates. [b] The *bglF* mutant strains used for assaying complementation were PPA543 and PPA547. These strains also carry mutant *crr* and *nagE* genes. [c] β-gal activity was measured in strain PPA547 which carries a chromosomal *bgl′-lacZ* transcriptional fusion and defective *bglF*, *crr*, and *nagE* genes. The shaded boxes are IIA^{bgl}. The open boxes are IIB^{bgl}. The cross-hatched boxes are IIC^{bgl}. The ● symbols mark phosphorlation sites. × symbols mark inactive phosphorylation sites (mutated).

BglF Mutant Proteins Lacking Different Phosphorylation Sites Can Combine To Complement Phosphotransfer to β-Glucosides and Negative Regulation of BglG Activity in Vivo. To substantiate our in vitro results, we examined the ability of BglF mutant proteins that complemented one another in vitro to complement in vivo. To test the ability of the mutant pairs to transfer β-glucosides into the cell while phosphorylating them, we assayed the ability of the coexpressed mutant proteins to complement strains defective in the *bglF* gene (PPA543 and PPA547, both also defective in the *crr* and *nagE* genes) and enable them to utilize β-glucosides. Complementation of the chromosomal mutation in the *bglF* gene by the plasmid-encoded BglF protein pairs was indicated by the formation of red colonies on MacConkey arbutin plates. Utilization of the β-glucoside arbutin depends on the ability of the plasmid-encoded BglF derivatives to phosphorylate and transport this sugar, which is then cleaved by the product of the unlinked locus *bglA*. Utilization of the β-glucoside salicin is prohibited in these strains due to the polarity of the mutation in *bglF* on the cotranscribed *bglB* gene, whose product preferentially cleaves phosphosalicin (*5*). The results are presented in Table 2. Consistent with our previously published results (*4*), neither H547R nor C24S alone could rescue the *bglF* strains for arbutin fermentation. However, the coexpressed pairs, C24S and IIBC^{bgl}, and H547R and IICA^{bgl}, behaved like wild-type BglF in their ability to convert the Arb$^-$ *bglF* strains to Arb$^+$.

BglF had previously been shown to exert its negative effect on operon expression by phosphorylating BglG, thus blocking the antiterminator function of the latter (*6*). To test whether the pairs of BglF mutant proteins phosphorylate BglG in vivo, we assayed their ability to negatively regulate BglG. To address this question, we made use of strain PPA547 (*4*), which carries a chromosomal *bgl′*−lacZ fusion (a fusion of the *bgl* promoter and transcription terminator to *lacZ*) and mutations in the *bglF*, *crr*, and *nagE* genes. Due to the mutation in the chromosomal *bglF* gene, BglG is not negatively regulated in this strain and therefore allows constitutive expression of *lacZ*.

The β-galactosidase levels of strain PPA547 containing plasmids which encode either the individually expressed BglF derivatives or the coexpressed BglF mutant proteins, grown in the absence and presence of the β-glucoside salicin, are given in Table 2. As expected, expression of plasmid-encoded wild-type BglF in PPA547 rendered *lacZ* expression inducible; i.e., β-galactosidase was produced only upon addition of salicin to the growth medium. Consistent with our previously published results (*4*), neither C24S nor H547R could regulate BglG and prevent constitutive expression of *lacZ* in this background. However, the coexpressed protein pairs C24S and IIBC^{bgl}, and H547R and IICA^{bgl}, each lacking one phosphorylation site, behaved like wild-type BglF and allowed *lacZ* expression only upon the addition of salicin.

These results, demonstrating that two BglF mutant proteins, one lacking site 1 and the other lacking site 2, can work together to implement β-glucosides phosphotransfer and BglG negative regulation, indicate not only that BglF can oligomerize but also that the oligomers are functional in vivo.

BglF Dimerizes via Its Hydrophobic IIC Domain. To demonstrate oligomerization of BglF directly, and to determine the oligomeric state of BglF, we examined its electrophoretic behavior as follows. Two plasmids, one containing the *bglF* gene cloned downstream of the phage T7 promoter and the other coding for T7 RNA polymerase, were cointroduced into cells. Following heat induction of T7 RNA polymerase and addition of rifampicin to shut off transcription by *E. coli* RNA polymerase, cells were pulse labeled with [^{35}S]methionine. When proteins were extracted with SDS at 100 °C, a single major radioactive band, corresponding to the BglF monomer (calculated MW of 66.4 kDa, apparent MW of ca. 71 kDa, see Table 3), was observed on

Table 3: Apparent Molecular Masses of BglF and Its Derivatives

BglF derivative	apparent MW (kDa)[a]	
	M[b]	D[c]
BglF	71	139
BglF-like	76	149
$IIBC^{bgl}$	52	106
$IICA^{bgl}$	62	109
IIC^{bgl}	45	95

[a] Molecular masses of radioactively labeled proteins were estimated from autoradiograms of SDS−polyacrylamide gels (Figure 3) using the Molecular program of the BAS1000 Bio-imaging analyzer. The values represent the average of six independent measurements. [b] M = monomer. [c] D = dimer.

autoradiograms of SDS−polyacrylamide gels (Figure 3A, lane 1). However, SDS extraction of proteins under mild conditions (30 °C, 30 min) led to the appearance of an additional band (Figure 3A, lane 2), which could correspond to a BglF dimer (calculated MW of 132.8 kDa, apparent MW of ca. 139 kDa, see Table 3).

To determine whether the band which appears only after mild extraction corresponds to the BglF homodimer or to a complex involving BglF and other proteins, we analyzed several lengthened and truncated BglF derivatives in a similar way. When a BglF derivative extended by few amino acids, designated a BglF-like protein, was extracted at 100 °C, a single major band, corresponding to a monomer, was observed (Figure 3A, lane 3). Again, an additional band was detected after mild extraction at 30 °C (Figure 3A, lane 4). The apparent molecular mass of this new band is ca. 149 kDa (Table 3), which corresponds to the size expected for a dimeric form of the BglF-like protein. Putative homodimers were also observed with the ^{35}S-labeled truncated protein $IIBC^{bgl}$, which contains the IIB and IIC domains of BglF (Figure 3B), and with the IIC^{bgl} domain alone (Figure 3C). The apparent molecular masses of the ^{35}S-labeled BglF derivatives and their respective homodimers, which correlate with the expected values, are given in Table 3. Dimers were not observed with $[^{35}S]IIB^{bgl}$ or with purified IIA^{bgl} and IIB^{bgl}, which were similarly extracted and analyzed (data not shown).

To determine unambiguously the identity of the labeled band that corresponds in size to a BglF homodimer, this band (Figure 3A, lane 2, band designated D) was excised, and the labeled protein was extracted from the gel slice by incubation in electrophoresis sample buffer for 2 h at 30 °C. After incubation of the extracted sample at 100 °C for 5 min, a single radioactive band, which corresponded in size to the BglF monomer, was detected by SDS−PAGE (Figure 3D, lane 1). Incubation of the sample at 30 °C before SDS−PAGE resulted in the dissociation of a certain fraction of the dimers to monomers (Figure 3D, lane 2). The last result highlights the limitations of this method and shows why it cannot be used to determine the exact ratio between monomers and dimers in the cell.

On the basis of all these results, we conclude that BglF forms homodimers. The membrane-spanning domain, IIC^{bgl}, is both necessary and sufficient for mediating dimerization.

The in vitro and in vivo results presented above indicated that BglF dimers can phosphorylate the BglF substrates. Therefore, we tested whether homodimers of the phosphorylated protein can be detected by this procedure. To this end, we labeled BglF in the in vitro phosphorylation system described above and analyzed it by SDS−PAGE following mild extraction. Homodimers of ^{32}P-labeled BglF were observed (Figure 3E, lane 1). Dimers were also observed with the phosphorylated BglF-like protein and $IICA^{bgl}$ (Figure 3E, lanes 2 and 3, respectively). Dimers of phosphorylated IIA^{bgl} and IIB^{bgl} were not observed by SDS−PAGE following mild extraction (data not shown). These results demonstrate unequivocally that phosphorylated BglF dimerizes and reinforce the finding that the hydrophobic IIC^{bgl} domain mediates BglF dimerization.

BglF Dimerization Is Not Modulated by β-Glucosides. The same active site of BglF transfers the phosphoryl group either to β-glucosides or to BglG, depending on whether β-glucosides are present outside the cell (*4*). One possible mechanism for ensuring delivery of the phosphoryl group to the correct entity would be induction of a conformational change in BglF by the sugar. In eukaryotes, receptor dimerization, often followed by its phosphorylation, is many times induced by a ligand. We therefore asked whether BglF dimerization is induced by β-glucosides. As shown in Figure 4, BglF dimers formed independently of the presence of the β-glucoside salicin in the growth medium during overproduction and ^{35}S labeling of BglF (compare lanes 1 and 2 in Figure 4A). Thus, even BglF that was not exposed to β-glucosides dimerizes, and the relative amount of BglF dimers does not change after β-glucoside addition.

We next asked whether β-glucosides can induce dissociation of existing BglF dimers. To answer this question, we added salicin to cells, following their pulse labeling with [^{35}S]methionine, together with cold methionine and removed aliquots for SDS−PAGE after various chase periods. The results of this experiment, presented in Figure 4B, demonstrate that the relative amount of BglF dimers was not affected by the addition of salicin. Moreover, [^{35}S]BglF dimers were also observed when salicin was added to pulse-labeled cells after they had been pelleted (Figure 4A, lane 3). Therefore, β-glucosides promote neither dimerization nor monomerization of BglF.

DISCUSSION

The results presented here demonstrate that BglF, the β-glucoside permease and the sensor of the *bgl* system, dimerizes. For phosphotransfer from PEP to the sugar or to BglG, the phosphoryl group needs to be transferred from the first to the second phosphorylation site of BglF. We have shown that this transfer can occur between subunits in the BglF dimer and that the transphosphorylated dimer is active in phosphorylation of the sugar and BglG. The demonstration that site 1 of one BglF mutant protein can transfer a phosphoryl group to site 2 of another BglF mutant protein in vitro establishes the first point. The ability of two coexpressed BglF mutant proteins, each lacking one phosphorylation site, to implement phosphotransfer to β-glucosides and to regulate BglG activity in vivo leads to the second conclusion. Moreover, efficient phosphoryl group transfer in vitro occurs only when the two types of BglF molecules, donor and recipient, are present together in the same membrane. Mixing membrane preparations, each of which contains one type of BglF molecule, led to negligible phosphotransfer. Thus, intermolecular phosphotransfer de-

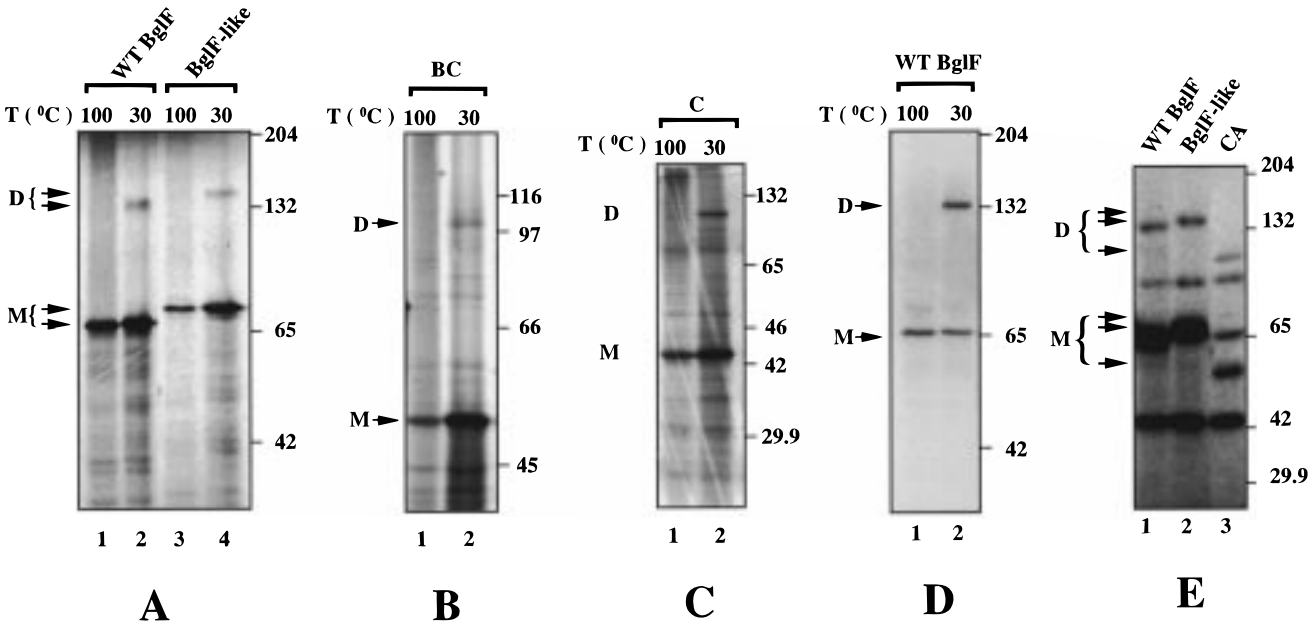

FIGURE 3: BglF dimerization is mediated by its membrane-bound IIC domain. (A−C) Overproduction of BglF and its derivatives was induced in *E. coli* K38 cells, and the cellular proteins were labeled with [^{35}S]methionine for 2 min (see Experimental Procedures). Proteins were extracted either at 100 °C for 5 min or at 30 °C for 30 min in electrophoresis sample buffer. Gradient (5 to 12.5%) SDS−polyacrylamide gels were used for the analysis of wild-type BglF and the BglF-like protein (A) and for the analysis of IIBC^{bgl} (B). IIC^{bgl} was analyzed on a 8 to 20% gradient tricine−SDS−polyacrylamide gel (C). (D) The gel slice containing the labeled band that corresponded in size to BglF dimers (panel A, lane 2) was excised, and the protein was extracted from it by incubation in electrophoresis sample buffer for 2 h at 30 °C. The gel-purified protein was divided into two samples, and one was further incubated for 5 min at 100 °C. Both samples were analyzed on a 5 to 12.5% gradient SDS−polyacrylamide gel. (E) Membrane fractions of cells that overproduce wild-type BglF, the BglF-like protein, or IICA^{bgl} were incubated with [^{32}P]PEP and purified enzyme I and HPr for 10 min. Proteins were extracted at 30 °C for 30 min in electrophoresis sample buffer. Samples were analyzed on a 5 to 12.5% gradient SDS−polyacrylamide gel. The autoradiograms are presented. Molecular masses of protein standards are given in kilodaltons. M = monomer; D = dimer. In panel E, phosphorylated enzyme I (migrating near the 65 kDa molecular mass marker) and IICB^{glc} (monomer, migrating near the 42 kDa molecular mass marker, and dimer) are also detected.

pends on dimer formation in the same membrane, presumably because the interaction between the subunits occurs between their membrane-spanning portions. This finding, together with the insensitivity of the dimers to reducing agent (all the SDS−PAGE analyses were carried out in the presence of 5% β-mercaptoethanol), suggests that the dimers might be held together by hydrophobic interactions within the membrane, which is similar to the case with the mannitol permease (see below).

The dimers can carry out both of the PEP-dependent phosphorylation functions of BglF: phosphorylation of β-glucosides and phosphorylation of the transcription antiterminator BglG. Nevertheless, it in not known whether the dimer is the only form that can carry out these reactions. Dimers are apparently not formed exclusively by phosphorylated BglF, because the extent of dimerization observed with phosphorylated BglF is not significantly different from the extent of dimerization seen in the total BglF content within the cell (see Figure 3). We therefore conclude that both phosphorylated and nonphosphorylated BglF dimerize. The method used for direct dimers detection (mild extraction followed by SDS−PAGE) does not allow us to determine the exact ratio between the dimeric and monomeric fraction of BglF in the cell.

The oligomeric structure of several other EIIs has been studied. The most extensively studied PTS permease in this respect is the *E. coli* mannitol permease, EII^{mtl}, which, like BglF, contains all three domains in one polypeptide. Dimerization of this permease has been demonstrated by various techniques (reviewed in ref *3*). EII^{mtl} embedded in the membrane seems to be predominantly a dimer (*34*). Dimerization of EII^{mtl} is mediated exclusively by its membrane-bound IIC domain (*21*, *35*). The EII^{mtl} dimer seems to be held together primarily by hydrophobic interactions between residues in this domain (*16*, *18*). Considerable evidence suggests that the dimer of EII^{mtl} is the more active form; both PEP-dependent and phosphoexchange activities of EII^{mtl} are catalyzed better by a dimer than by a monomer (reviewed in ref *3*). IIAB^{man}, the cytoplasmic constituent of the *E. coli* mannose-specific EII that contains phosphorylation sites 1 and 2, was found to exist as a very stable dimer (*36*). Intersubunit phosphotransfer has been demonstrated to occur in the IIAB^{man} dimer (*37*, *38*). The *E. coli* glucose permease, which contains only domains IIC and IIB (IICB^{glc}), and therefore only phosphorylation site 2 (site 1 resides on a separate cytoplasmic component, IIA^{glc}), was first purified as a dimer which, together with four molecules of IIA^{glc}, precipitated with antibodies raised against IICB^{glc} (*39*). However, later experiments suggested that the catalytically active glucose permease is a monomer and that the dimeric IICB^{glc} might have been formed in the course of protein purification (*40*). BglF is now added to the list of EIIs which dimerize. BglF dimers are shown here to catalyze PEP-dependent phosphorylation of both a sugar and a regulatory protein, i.e., BglG. With other EIIs, only the ability of the dimeric forms to catalyze PEP-dependent sugar phosphorylation and phosphoexchange activities has been shown.

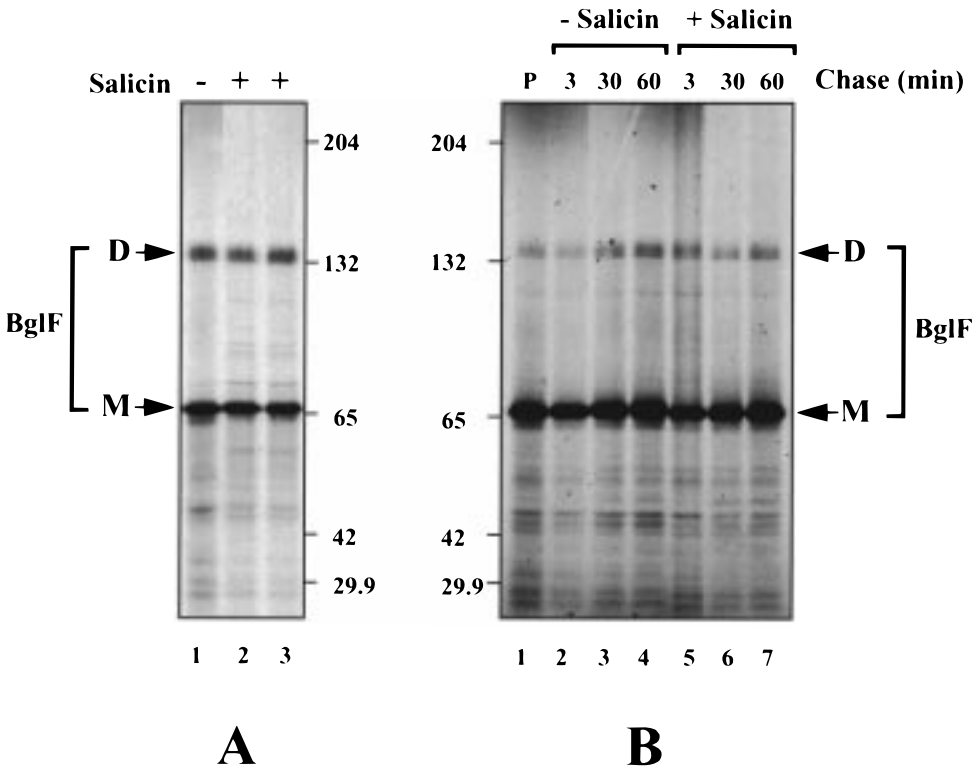

FIGURE 4: BglF dimerization is not modulated by β-glucoside. Overproduction of BglF was induced in *E. coli* K38 cells, and the cellular proteins were pulse labeled with [^{35}S]methionine for 2 min (see Experimental Procedures). (A) In lane 1, growth and overproduction occurred in the absence of salicin. In lane 2, cells were grown in the absence of salicin but BglF overproduction was carried out in the presence of 0.2% salicin. In lane 3, growth and overproduction occurred in the absence of salicin, but after harvesting, cells were resuspended in a buffer containing 0.2% salicin. (B) After pulse labeling, unlabeled methionine was added with (+) or without (−) 0.2% salicin and aliquots were removed after the chase periods written above the lanes. P = pulse labeling without chase. Proteins were extracted at 30 °C for 30 min in electrophoresis sample buffer. Samples were analyzed on 5 to 12.5% gradient SDS−polyacrylamide gels which were subsequently analyzed by autoradiography. Arrowheads indicate the positions of the dimeric (D) and monomeric (M) forms of BglF. Molecular masses of protein standards are given in kilodaltons.

Several explanations, which are not mutually exclusive, have been suggested to rationalize EIIs dimerization. (1) Subunit interaction may result in the formation of a pore that provides a transport route for the sugar across the membrane (*25*). On the basis of simple geometric considerations, an oligomer, minimally a dimer, seems to be required to form a channel large enough to admit the sugar (*15*). (2) Cooperative binding site interactions may facilitate rapid sugar transport (*21*, *25*). This idea is supported by the finding that EII^{mtl} contains one high-affinity and one low-affinity mannitol binding site per dimer (*41*). (3) Dimers are required for catalyzing efficient phosphotransfer from site 1 of one molecule to site 2 of another molecule in those EIIs in which both phosphorylation sites exist on the same protein molecule. Intrasubunit phosphotransfer might be inefficient in these permeases. This idea is supported by the finding that the *N*-acetylglucosamine permease (EII^{nag}) and BglF, both of which contain sites 1 and 2 in a single polypeptide, combine with IICB^{glc} in a strain lacking IIA^{glc} to carry out glucose and α-methyl glucoside transport and phosphorylation (*8*, *42*). Presumably, functional heterodimers are formed in the membrane, at least transiently, between EII^{nag} or BglF and IICB^{glc} to allow phosphotransfer to occur. The results reported here, which indicate that BglF dimerizes and that intersubunit phosphotransfer occurs within these dimers, reinforce this idea. Nevertheless, intersubunit phosphotransfer has been demonstrated in all cases, including the case of BglF, by the use of pairs of mutant proteins that can complement one another. The question arises as to whether this intersubunit pathway is indeed the major route for phosphotransfer within the wild-type protein. On the basis of the low PEP-dependent activity of dissociated EII^{mtl} (*43*) and the quantitation of in vivo complementation activities of EII^{mtl} mutant variants (*24*), it was concluded that this is indeed the case for EII^{mtl}. Future studies will determine whether this conclusion can be extended to other EIIs.

BglF is the only EII known to catalyze phosphorylation of a transcription regulator in addition to a sugar. Phosphorylation of the sugar and the regulator are mutually exclusive events; in the absence of β-glucosides, BglF phosphorylates the regulator, BglG, and upon addition of β-glucosides, BglF dephosphorylates BglG and phosphorylates the sugar. Therefore, β-glucosides trigger BglF to switch from being a BglG kinase to being a BglG phosphatase and sugar phosphotransferase. What is the nature of the change that BglF undergoes when it switches between the alternative enzymatic activities? How does the sugar induce this change? In eukaryotes, ligands often induce receptor dimerization. Our results suggest that BglF dimerization is not modulated by β-glucosides; the sugar does not seem to cause monomeric BglF to dimerize, nor does it seem to impel dissociation of BglF dimers. Moreover, the ability of the dimers to carry out phosphorylation of both the sugar and BglG does not suggest that a switch between monomeric and dimeric forms determines which activity predominates.

Other mechanisms that can explain the sugar-driven signal transduction pathway and the observation that the same active site phosphorylates both β-glucosides and BglG are currently being investigated.

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