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Interplay between the Membrane-Associated UhpB and UhpC Regulatory Proteins

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Expression of the *Escherichia coli uhpT* gene, encoding the sugar phosphate transport protein, is induced by extracellular glucose-6-phosphate and requires the function of the *uhpABC* regulatory genes. The UhpA and UhpB proteins are related to the response-regulator and sensor-kinase proteins of two-component regulatory systems, whereas the UhpC protein is related to UhpT and homologous transport proteins. To investigate the role of segments of the membrane-associated UhpB and UhpC regulatory proteins, a series of mutations were constructed in vitro by insertion of a 12- or 24-bp oligonucleotide linker at 44 sites within the *uhpABCT* locus. The effect of these mutations on regulation of a *uhpT-lacZ* transcriptional reporter was assayed with the mutated *uhp* alleles in single copy on the chromosome. All but one of the insertions in *uhpA* or *uhpT* were inactive for transcription activation or transport, respectively. In contrast, about half of the insertions in *uhpB* and *uhpC* retained Uhp expression, and insertions at four sites in *uhpB* and at one site in *uhpC* conferred high-level constitutive expression. The constitutive mutants in UhpB resulted from insertions in the nonpolar amino-terminal half of the protein, and all insertions in that half of UhpB affected Uhp expression in some manner, which suggests that the transmembrane segments of UhpB might negatively regulate the kinase activity of the carboxyl portion. The constitutive behavior of all but one of these *uhpB* alleles was dependent on the presence of active forms of both UhpA and UhpC, which suggests that UhpB and UhpC act jointly as a complex in the signaling process.

The products of the *uhpABC* regulatory genes control the induction of the synthesis of the *Escherichia coli* sugar-phosphate transporter, UhpT, by extracellular glucose-6-phosphate (Glu6P) (20, 21). Genetic studies have led to the proposal that the UhpA protein specifically activates transcription at the *uhpT* promoter in response to a transmembrane signaling event mediated by the UhpB and UhpC proteins (4). Sequence comparisons showed that UhpA and UhpB are related to the response-regulator and sensor-kinase proteins of two-component regulatory systems, respectively (7, 20). Two-component regulatory systems are widespread in bacteria and mediate responses to environmental signals by a process that involves phosphate transfer reactions between the component proteins (reviewed in references 11 and 17). Response-regulator proteins, such as UhpA, CheY, OmpR, and NtrC, possess a conserved amino-terminal module of 120 to 130 amino acids which contains the aspartyl residue that is phosphorylated. The existence of distinct subfamilies of response regulators is evident from sequence homologies within both the phosphorylation modules and the carboxyl-terminal domains, which are implicated in DNA binding and transcription activation. UhpA belongs to the subfamily that includes the regulatory proteins NarL, FixJ, DegU, and ComA (2, 10).

The sensor-kinase proteins, such as UhpB, CheA, EnvZ, DegS, and NtrB, have similar sequences in a 200-amino-acid segment that is often at the carboxyl end of the protein and includes a glycine-rich ATP-binding motif and two conserved segments flanking invariant histidine and asparagine residues (reviewed in reference 11). Several of these proteins transfer phosphate from ATP to their conserved histidine residue in a process that is regulated in response to an appropriate environmental signal. Transfer of phosphate from the histidine on the sensor-kinase protein to the aspartyl residue on the cognate response-regulator protein results in the activation of that protein, which usually involves its function to stimulate specific gene transcription. Many sensor-kinase transducer proteins are predicted to contain two membrane-spanning segments in their amino-terminal portion and thereby expose a substantial periplasmic domain that could bind their extracellular signal molecule. The bipartite UhpB protein differs from this usual structure, in that the amino half of UhpB (residues 1 to 273) is highly hydrophobic and is likely to cross the cytoplasmic membrane 6 to 10 times (4). The polar carboxyl half of UhpB (residues 274 to 500) contains the kinase motifs and lies in the cytoplasm.

The UhpC regulatory protein has similarity in length and transmembrane topology and about 30% amino acid sequence identity to UhpT and related transporters (4). This relatedness led to the suggestion that UhpC serves as the receptor for external Glu6P. The requirement for a third protein in the signaling process suggests that Uhp regulation might differ from the mechanisms that control other two-component systems.

We describe the construction of linker insertion mutations in the *uhp* genes that provide information about the involvement of segments of the *uhpB* and *uhpC* products in Uhp regulation. The properties of these mutants indicate that the membrane-embedded, amino-terminal half of UhpB might play a negative role in the control of *uhp* expression, while the epistatic relationships deduced from combining mutant

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alleles of *uhpB* and *uhpC* suggest the existence of a membrane-associated UhpB-UhpC complex in the signaling process.

MATERIALS AND METHODS

Plasmids, phage, and bacterial strains. The mutations isolated in this study are listed in Table 1. The host strain for all plasmid constructions was XL-1 Blue [*recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 lac*/F' proAB $lacI^qZ\Delta M15$ *zzf*::Tn*10*] (Stratagene, La Jolla, Calif.). Plasmid pMI29 (4) was constructed by cloning the 6.0-kb *Eco*RI-*Bam*HI fragment from pRJK10 (19), containing the entire *uhp* region, into pBluescript KS^+ (Stratagene) that had been modified by removal of several restriction sites in the multiple cloning site. The isolation of a series of insertions of a kanamycin resistance cassette (*aph*) in the *uhp* region in plasmid pMI29 was described previously (4). Expression and regulation of the *uhpT* promoter were quantified by measurement of the level of β-galactosidase encoded by a *uhpT-lacZ* operon fusion (9) carried as a single lysogen of phage λRZ5-*uhpTp-lac*[Km] in strain RK9338 [Δ(*argF-lac*)*U169 araD139 thi gyrA219 relA rpsL150 non polA1* Δ(*ilvBN-uhpABCT'*)*2095 zig621*::Tn*10*], as previously described (4).

DNA manipulations. Routine recombinant DNA procedures were performed by standard methods (13) or the conditions recommended by the manufacturer of the product.

Construction of 4-codon linker insertion mutations. Each kanamycin resistance cassette inserted in the *uhp* locus was flanked by a restriction site linker. In most cases, religation after digestion with *Pst*I resulted in excision of the kanamycin resistance determinant and left a 12-bp insertion (5'-GACCTGCAGGTC). In a few cases, excision with *Pst*I left a 24-bp insert, and excision with *Sal*I left a 12-bp insert. Plasmids carrying two linker insertions were constructed by exchanging the 2.4-kb *Bst*EII-*Bgl*II fragment (positions 1988 to 4416 of the *uhp* sequence [4] [GenBank accession no. M89479]) from a plasmid carrying a linker insertion in *uhpA* or *uhpB* with the same fragment from a plasmid carrying a linker insertion in *uhpC*. The presence of both insertion mutations was verified by the release of the appropriately sized DNA fragment upon digestion with *Pst*I.

Transfer of mutations to the chromosome. All pMI29 derivatives carrying linker insertions were transferred to the chromosome of the *polA* strain RK9338, in which the plasmids cannot replicate. Ampicillin-resistant transformants resulted from integration of the plasmids by homologous recombination between *uhpT* sequences present on plasmid and chromosome.

Assays of Uhp function and regulation. Uhp function was tested by growth on minimal medium with 0.2% fructose-6-phosphate as a carbon source, relative to growth with 0.2% glucose. For determination of β-galactosidase activities, cells were grown to mid-log phase at 37°C in minimal medium supplemented with 0.4% glycerol and 5% casein hydrolysate in the presence or absence of 0.34 mM Glu6P, as previously described (4). Enzyme activities were assayed by the rate of hydrolysis at 37°C of *o*-nitrophenyl-β-D-galactopyranoside, as previously described (4, 9). Enzyme activities for each strain are averages of at least triplicate determinations.

RESULTS

Isolation of *uhp* linker insertion mutations. We described previously the isolation of a series of 44 insertions of a kanamycin resistance cassette in the *uhp* genes (4). Removal of each kanamycin resistance cassette by religation after cleavage at sites at the ends of the cassette with the appropriate restriction enzyme (*Pst*I or *Sal*I) left a 12-bp linker insertion (in two cases, a 24-bp insertion was obtained by cleavage with *Pst*I). This method allowed the isolation of 46 linker-insertion mutations in the *uhp* coding region, of which 4 were in *uhpA*, 15 were in *uhpB*, 13 were in *uhpC*, and 14 were in *uhpT*. Table 1 presents the location of each insertion and the amino acids that were added to the protein product. Although the same nucleotide sequence was inserted, the encoded amino acids depended on the reading frame. Our genetic nomenclature identifies each mutation by the location of the amino acid residue in the wild-type sequence that precedes the site of insertion, e.g., the mutated gene *uhpA15*::*4* carries a 4-codon insertion and encodes the UhpA15::4 protein with the insertion of the amino acid sequence Asp-Leu-Gln-Val between amino acids 15 and 16 of UhpA. To determine the effect of the linker insertions on Uhp function, each mutant plasmid was integrated in single copy at the chromosomal *uhp* locus by homologous recombination.

When Uhp function was assayed by growth on Fru6P as carbon source, all 4 of the 4-codon insertions in *uhpA* and 11 of the 12 insertions in *uhpT* were inactive (Table 1). The single Uhp^+ insertion, UhpT189::4, is predicted to be in a short periplasmic loop (4, 8). Insertions that are predicted to occur in other extramembranous loops of UhpT (at positions T285 in a short periplasmic loop; T153 and T157 in a short cytoplasmic loop; and T217, T221, and T249 in the large, central cytoplasmic loop) resulted in loss of function.

In light of the susceptibility of UhpA and UhpT function to disruption by 4-codon insertions, it was surprising that roughly half of the linker insertions in UhpB (8 of 15) and UhpC (6 of 13) allowed full or partial growth on Fru6P. The two *uhpC* insertions that were predicted to occur in transmembrane segments were Uhp^-. In contrast, of the nine linker insertions in the hydrophobic amino-terminal half of UhpB (residues 1 to 273), six displayed a Uhp^+ growth phenotype. There was no correlation between the identity of the inserted amino acids and the Uhp phenotype. Thus, the function of the UhpB and UhpC regulatory proteins was less susceptible to disruption by the insertion of four polar amino acids than was that of the UhpA and UhpT proteins.

Effect of insertion mutations on *uhpT-lac* expression. The effect of the 4-codon insertions in *uhpA*, *uhpB*, and *uhpC* on the level and regulation of *uhpT* expression was determined by use of a *uhpT-lacZ* reporter construct carried as a single lysogen of a λ transducing phage. Insertions in *uhpT* were not examined because previous work has shown that *uhpT* function has no direct effect on its regulation (4, 15). Cells were grown in the absence and presence of inducing levels of Glu6P, and the specific activity of β-galactosidase was determined for multiple independent isolates of each strain. Of the insertions affecting UhpA, three (at positions A15, A169, and A189) were completely devoid of *uhpT-lacZ* expression under all conditions tested (Table 1). Insertion UhpA132::4 exhibited a very low level of expression under inducing conditions (2% of induced wild type).

Insertions in *uhpB* conferred three different regulatory phenotypes (Table 1 and Fig. 1). All of the Uhp^- insertions (4-codon insertions at positions B87, B240, B288, B345,

TABLE 1. Effect of *uhp* linker insertions on Uhp phenotype and *uhpT-lacZ* expression

Position of insertion in:		Inserted amino acids	Uhp phenotype[a]	β-Galactosidase activity[b] with the following allele[c]:					
Nucleotide sequence (bp)	Amino acid sequence			*uhpC*$^+$		*uhpC91::4*		*uhpC91::8*	
				−	+	−	+	−	+
Wild type			+	2	1,550	3,800	3,000	13	11
143	A15	DLQV	−	1	1	1	1	3	1
495	A132	DLQV	−	1	30	110	145	3	2
605	A169	TCRS	−	2	2	1	1	2	1
666	A189	DLQV	−	2	2	2	2	2	1
869	B60	DLQV	+	106	1,540	2,700	2,140	28	23
885	B65	GPAGP[d]	+	46	860	57	33	184	139
949	B87	IRRR	−	35	30	127	75	150	115
1143	B151	GPAGP	+	2,260	1,700	2,530	1,620	2,515	1,940
1234	B182	TCRS	+	2,330	2,150	3,020	2,260	16	13
1254	B188	*stop*	−	78	54	ND	ND	ND	ND
1313	B208	DLQV	+	2,770	2,210	ND	ND	5	4
1410	B240	GPAGP	−	27	25	65	44	140	110
1435	B250	TCRS	+	1,035	2,155	3,105	2,135	12	11
1553	B288	DLQV	−	45	32	140	115	105	75
1725	B345	DLQV	±	115	143	90	57	110	85
1850	B387	DLQV	+	62	1,755	3,020	2,025	77	50
1921	B411	TCRS	−	57	43	70	86	125	105
2107	B473	TCRS	−	10	21	ND	ND	ND	ND
2156	B489	DLQV	−	2	17	ND	ND	ND	ND
2319	C41	GPAGP	−	5	6				
2470	C91::4	PSTD	+	3,945	3,355				
2470	C91::8	PSTCRSTD	−	10	9				
2621	C141	DLQV	−	7	7				
2871	C224	GPAGP	±	7	300				
2912	C238	DLQV	+	2	1,050				
2922	C241::4	IRRR	+	4	1,225				
2922	C241::8	IRRPAGRR	±	2	700				
3119	C307	DLQV	−	ND	ND				
3280	C361	TCRS	−	8	8				
3395	C399	DLQV	−	10	9				
3409	C404	TCRS	−	11	14				
3509	C437	DLQV	+	2	780				
3603	Tp		−						
3797	T42	*stop*	−						
4129	T153	DLQV	−						
4140	T157	TCRS	−						
4236	T189	TCRS	+						
4274	T201	GPAGP	−						
4320	T217	TCRS	−						
4332	T221	TCRS	−						
4418	T249	IRRR	−						
4491	T274	TCRS	−						
4526	T285	GPAGP	−						
4667	T332	GPAGP	−						
4767	T366	TCRS	−						
4952	T427	GPAGP	−						

[a] +, positive; −, negative; ±, intermediate.
[b] β-Galactosidase activity is given in $mOD_{415} \times minute^{-1} \times OD_{590}\ culture^{-1}$.
[c] − and +, the absence or presence, respectively, of Glu6P during growth. ND, not determined.
[d] G indicates that the amino acid residue preceding the site of insertion was converted to a glycine.

B411, B473, and B489) and the stop codon inserted at position B188) exhibited low, uninducible levels of β-galactosidase. As was previously found for *uhpB* deletion mutations (4), the uninduced level of β-galactosidase was substantially higher than that of uninduced wild-type cells or of the *uhpA* insertion mutants for all these *uhpB* insertion mutants, except UhpB473::4 and UhpB489::4. Insertion UhpB345::4, which conferred weak growth on Fru6P, resulted in constitutive production of β-galactosidase at about 10% of the induced wild-type level. None of the seven Uhp$^+$ insertions in *uhpB* displayed wild-type regulation. Three insertions (at positions B151, B182, and B208) exhibited high and constitutive levels of Uhp expression; the slight reduction in the presence of Glu6P is probably the result of

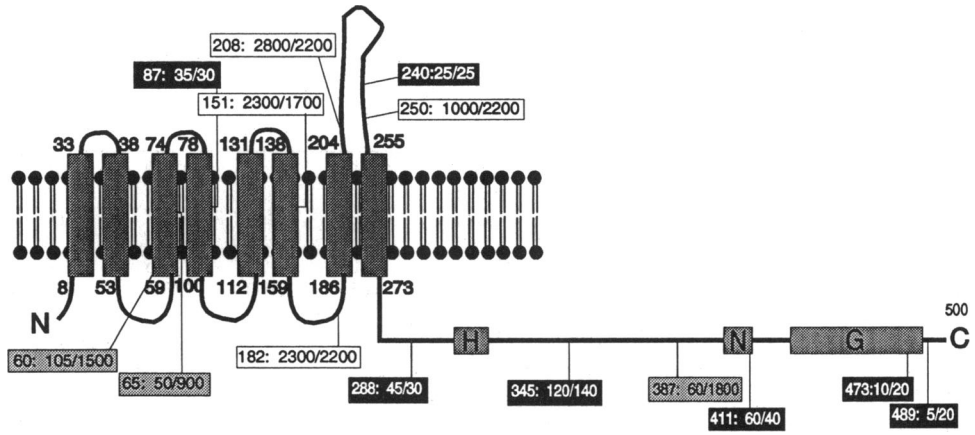

FIG. 1. Topological model for UhpB and location of linker insertions. The transmembrane topology, indicated by the location of the shaded transmembrane segments, is based on hydropathy analysis, which predicts as many as 10 membrane-spanning segments (4, 7), and *uhpB-phoA* fusions, which are consistent with the presence of at least six membrane-spanning regions. In this mode, eight transmembrane segments are presented, because this is the only topology which fits all of the PhoA fusion results, maintains electroneutrality of the membrane-spanning segments, and has a preponderance of positive charge at cytoplasmic loops. In the carboxyl-terminal half of the diagram are shaded boxes indicating the location of conserved sequences flanking the invariant histidine (H) and asparagine (N) residues and the glycine-rich ATP-binding motif (G) (11). The locations of the linker insertions in UhpB are indicated by the boxes, which give the amino acid residue before the insertion and the uninduced and induced levels of β-galactosidase taken from Table 1. The mutants in black boxes are negative for Uhp expression; those in white boxes are constitutive; those in shaded boxes are inducible.

catabolite repression. The other four Uhp$^+$ insertions in UhpB (at positions B60, B65, B250, and B387) possessed elevated basal levels of Uhp expression and were induced by Glu6P to near the induced wild-type level. These results showed that mutational alterations affecting the nonpolar amino-terminal half of UhpB can result in partial or full constitutive expression of *uhpT*, suggesting that the membrane-embedded segment of UhpB plays a negative role in regulating UhpB function.

All of the Uhp$^-$ insertions in UhpC (at positions C41, C141, C361, C399, and C404) were defective in activation of *uhpT-lacZ* transcription (Table 1). Most of the Uhp$^+$ insertions (C224, C238, C241, and C437) showed nearly normal Glu6P-inducible expression of β-galactosidase. Of the two insertions at position C91, the 4-codon insertion *uhpC91::4* was Uhp$^+$ and conferred high-level constitutive expression of β-galactosidase, but the 8-codon insertion at the same site, *uhpC91::8*, was Uhp$^-$ and incapable of activating *uhpT-lacZ* transcription. The site of the UhpC91 insertions is predicted to be in the first cytoplasmic loop of UhpC. The 4- and 8-codon insertions at UhpC241 conferred similar inducible Uhp$^+$ expression, showing that the additional amino acids in an 8-codon insertion do not necessarily eliminate UhpC function.

Epistatic relationships of UhpC and UhpB. To test the effect that the presence of different *uhpC* alleles might have on the regulatory phenotype of insertions in *uhpA* or *uhpB*, a series of double mutations were constructed in vitro by restriction fragment exchange and were analyzed for regulatory behavior. Epistatic relationships among genes affecting a signaling pathway might define the order of action of the components in that pathway when the phenotype conferred by a mutation in one gene is unaffected by the genotype of a component that acts earlier in that pathway. Double mutants which combined the allele for the constitutive UhpC91::4 form or the negative UhpC91::8 form with most of the 4-codon insertions in *uhpA* or *uhpB* were constructed. The resultant double mutations were transferred to the chromosomal *uhp* locus and assayed for their effect on expression and regulation of the *uhpT-lacZ* reporter gene (Table 1).

The very low and uninducible β-galactosidase expression in the presence of insertion mutations in *uhpA* (at positions A15, A169, and A189) was not changed by the presence of the various *uhpC* alleles. The *uhpA132::4* allele, which displays slight induction by Glu6P, showed constitutive but low-level expression in combination with the constitutive *uhpC91::4* allele and negative expression in combination with the negative *uhpC91::8* allele, consistent with the requirement for UhpA activator function for the expression of any altered regulation conferred by the UhpC variants.

When different mutant alleles of *uhpB* were combined with the *uhpC91* alleles, four different regulatory responses were seen (Table 1). Strains carrying the *uhpB* alleles that resulted in negative expression of β-galactosidase (at positions B87, B240, B288, B345, and B411) were negative for Uhp expression whether combined with the inducible *uhpC*$^+$ allele, the constitutive *uhpC91::4* allele, or the negative *uhpC91::8* allele. Strains carrying the *uhpB* alleles which conferred inducible Uhp expression in the presence of *uhpC*$^+$ (at positions B60, B250, and B387) displayed high-level constitutive expression of β-galactosidase when combined with the constitutive *uhpC91::4* allele but displayed essentially negative expression when combined with the negative *uhpC91::8* allele. Strains carrying the *uhpB151::4* allele displayed high-level constitutive expression of β-galactosidase regardless of which *uhpC91* allele was present. Surprisingly, strains carrying the *uhpB182::4* and *uhpB208::4* alleles displayed high-level constitutive expression of β-galactosidase in combination with the inducible *uhpC*$^+$ allele or the constitutive *uhpC91::4* allele, but gave low and uninducible expression in the presence of the negative *uhpC91::8* allele. Thus, the presence of an active form (inducible or constitutive) of UhpC cannot overcome the loss of Uhp expression conferred by Uhp$^-$ forms of UhpA or UhpB, suggesting that UhpC precedes UhpA and UhpB in the signaling pathway. However, the fully or partially constitutive regulatory behavior conferred by most Uhp$^+$ insertions in UhpB was

TABLE 2. Expression of *uhpT-lacZ* in strains carrying insertions in *uhpC* and *uhpA* or *uhpB*

uhpC allele	β-Galactosidase activity[a] in the presence of listed *uhp* allele[b]:											
	A⁺B⁺		*A15::4*		*B151::4*		*B182::4*		*B208::4*		*B250::4*	
	−	+	−	+	−	+	−	+	−	+	−	+
C⁺	2	1,550	1	1	2,260	1,700	2,330	2,150	2,770	2,210	1,035	2,155
C41	5	6	2	1	1,920	1,450	10	8	3	2	8	8
C91::4	3,945	3,355	1	1	2,530	1,620	3,020	2,260	ND	ND	3,105	2,135
C91::8	10	9	3	1	2,510	1,940	16	13	5	4	12	11
C141	7	7	1	1	2,790	2,220	10	9	5	4	12	14
C224	7	300	3	1	2,425	1,965	405	1,160	2,785	2,420	1,135	1,460
C361	8	8	ND	ND	1,865	1,480	8	6	3	2	12	13
C399	10	9	1	1	2,190	1,700	5	3	4	3	8	7
C404	11	14	1	3	3,100	2,110	7	12	3	5	13	26
Δ(C41-C437)	10	9	1	1	2,005	1,760	10	9	3	2	20	18

[a] β-Galactosidase activity is given in $mOD_{415} \times minute^{-1} \times OD_{590}\ culture^{-1}$.
[b] − and +, the absence or presence, respectively, of Glu6P during growth. ND, not determined.

dependent on the presence of an active (inducible or constitutive) form of UhpC.

Effect of UhpC on constitutive UhpB mutants. To verify that an active form of UhpC is needed for UhpB function, we constructed double mutants combining each constitutive *uhpB* allele with most of the linker insertions in *uhpC* and with a *uhpC* deletion that removed UhpC residues 41 to 437 (Table 2). As before, the constitutive *uhpB151::4* allele showed high-level constitutive expression of β-galactosidase when combined with any of the *uhpC* alleles. In contrast, and in agreement with the results presented above, insertions at positions B182, B208, and B250, which showed constitutive Uhp expression in combination with the wild-type *uhpC⁺* allele, displayed very low and noninducible Uhp expression when combined with any of the negative *uhpC* linker insertions or the deletion mutation. A partially active form of UhpC, encoded by the *uhpC224::4* allele, allowed expression of the constitutive phenotype of the UhpB insertions, although the maximal level of expression was reduced in some of these strains.

DISCUSSION

Although two of the Uhp regulatory proteins are related in sequence to members of two-component regulatory systems, the Uhp regulatory system differs from most two-component systems in its requirement for a third regulatory protein, UhpC, and in the predicted transmembrane topology of the sensor-kinase protein, UhpB, which lacks the large periplasmic loop present in many transmitter proteins. The transmembrane signaling process that controls Uhp expression might reflect these differences in structure. To study the role in regulation of portions of the Uhp regulatory proteins, we made use of a series of mutations constructed in vitro by the insertion of a 12- or 24-bp oligonucleotide linker into the *uhp* locus. Advantages of this type of mutational analysis include the ease with which these mutations are created and localized in the nucleotide sequence and, as previously described (4), their utility for subsequent genetic manipulations, such as the generation of deletion mutations and gene fusions. Since their isolation does not involve selection for any phenotype, these mutations provide a general method to probe the contribution of specific portions of a protein to its function. The linkers used in this study inserted four or eight amino acids whose identity depended on the specific reading frame but which added at least one charged residue. These insertions are expected to substantially perturb the local protein conformation, perhaps more so than would any single amino acid substitution, and thereby show more clearly the functional requirement for the disrupted portion of the protein than would be possible by random chemical mutagenesis.

The effect of the linker insertions on Uhp regulation revealed two unexpected results that have implications about the domain structure and interactions of the Uhp regulatory proteins, although specific conclusions must await biochemical determinations of the activity, stability, and cellular location of the wild-type and mutant proteins. Many insertions in *uhpB* and *uhpC* retained Uhp expression, whereas most insertions in *uhpA* and *uhpT* resulted in loss of function. Also, mutants of UhpB which confer constitutive expression are still dependent on the presence of an active form of UhpC.

Since UhpC and UhpT are similar in sequence and transmembrane topology (4), they are likely to insert into the membrane in a similar manner and may both have binding sites for sugar phosphates. About half of the insertions in UhpC were able to confer normal induction by Glu6P. The three insertions in the predicted central cytoplasmic loop in UhpC were Uhp⁺ with inducible behavior, whereas all three insertions in the central loop of UhpT were Uhp⁻. This result suggests that the central cytoplasmic loop is important for UhpT transport function but not for UhpC signaling activity. The insertion of four amino acids at residue 91 in UhpC resulted in high-level and constitutive expression of *uhpT-lacZ*, which was still dependent on both UhpA and UhpB function. Therefore, this lesion does not bypass the normal signal transduction pathway, suggesting that the cytoplasmic loop of UhpC that contains amino acid 91 might be involved in signaling.

The null phenotype of a *uhpB* deletion confers uninducible but slightly elevated expression over that of the uninduced wild type, up to about 2% of the induced wild type (4). A simple explanation of this behavior is that UhpB possesses both protein kinase and phosphatase activities and that the phosphatase function is needed to reverse adventitious phosphorylation of UhpA by other protein kinases when in the uninduced state. The same uninducible but elevated expression was found with most of the Uhp⁻ linker insertions in *uhpB*, except for the insertions near the carboxyl end of the protein. Both UhpB473::4 and UhpB489::4 exhibited slight inducibility and a low basal expression near that of wild

type. It is possible that these UhpB variants retain phosphatase activity but lack either the kinase activity or the ability to couple kinase activation to the presence of the inducing signal.

All linker insertions in *uhpB* that resulted in high-level constitutive expression of the *uhpT-lacZ* reporter affected the nonpolar amino-terminal half of the protein, and, conversely, all mutations in that half of UhpB had a significant regulatory effect (shown schematically in Fig. 1). This finding that mutational distortion over a considerable length of the transmembrane portion of UhpB (including residues 151 to 250, where insertions result in constitutive expression) resulted in increased Uhp expression comparable to receptor occupancy by Glu6P suggests that this part of UhpB acts in a negative manner to block UhpB function. A situation in which the transmembrane portion of UhpB does not have a direct inhibitory function, but serves to transmit a conformational change resulting from ligand binding to an external domain, as in models for the chemotactic receptor-transducer proteins, would be expected to have some external sites where mutation could confer constitutive expression, but this would most likely occur at only a small number of sites, unlike the observed situation with UhpB. The negative, inhibitory action of part of a signaling protein on a protein kinase activity is seen, for example, in the eukaryotic protein kinases A and C, in which a segment of either another protein or the same polypeptide chain, respectively, acts as a pseudosubstrate by binding to and inhibiting the kinase active site (1, 3, 18). Removal of the pseudosubstrate portion from protein kinase C by mutation or proteolysis results in activation of kinase function and independence from control by normal effectors (reviewed in reference 14). There is no obvious relatedness between sequences in the amino-terminal half of UhpB and the phosphorylation domain of UhpA that might indicate a pseudosubstrate site. The putative regulation by the transmembrane portion of UhpB would most likely occur through the cytoplasmic loops but would be affected by the conformation of the entire domain. This regulation could affect autophosphorylation of UhpB or a later stage of protein recognition or phosphate transfer to UhpA, or even protein phosphatase activity. Further work is necessary to demonstrate whether and how the transmembrane segment acts in a negative manner on UhpB activity.

Whereas some two-component systems appear to recognize the environmental signal through the sensor-kinase component, increasing numbers of others are found to use additional proteins. The PhoR kinase is negatively regulated by the high-affinity Pst phosphate transport system in response to the presence of external P_i. The mechanism by which Pst affects PhoR activity appears to be coupled through the product of the *phoU* gene in the *pst* operon (12, 16). Unlike UhpC, PhoU appears to act as a negative regulator of kinase activity since mutations in *phoU* result in constitutive expression of the Pho regulon. Another example is the phosphoglycerate transporter of *Salmonella typhimurium*, PgtP, which is related in sequence to UhpT and whose synthesis is regulated by a three-component system. PgtA is a positive activator of *pgtP* transcription, and both PgtB and PgtC are membrane-bound proteins required for signal transduction (22, 23). PgtA and PgtB possess regions homologous to receiver and transmitter domains, respectively (11). Genetic data support a role for all three components in proper regulation, but, in contrast to the Uhp system, deletion of both *pgtB* and *pgtC* results in constitutive expression of *pgtP* (5). This observation led to the proposal that PgtB and PgtC act to sequester or change the conformation of PgtA upon inducer binding, rather than to modify PgtA chemically (5).

The second unexpected finding in this study is the dependence of most constitutive UhpB mutants on the presence of an active form of UhpC. The large number of sites in UhpB at which insertions confer constitutive expression is consistent with the high frequency of constitutive mutants that allow growth on noninducing sugar phosphates (6). However, since most of the constitutive *uhpB* insertions were still dependent on the presence of an active form of *uhpC*, this part of UhpB cannot be the major site of alteration in constitutive pseudorevertants of *uhpC* null mutations (4, 6, 18a). UhpC-independent constitutive mutations have been located at numerous sites in the carboxyl-terminal half of UhpB (18b). The dependence of constitutive UhpB mutants on UhpC function suggests either that UhpC is essential for the function, stability, or membrane insertion of UhpB or that UhpB and UhpC form an interactive signaling complex. The requirement for UhpC for membrane insertion or stability of UhpB is less likely, because of the existence of constitutive UhpC-independent mutants in UhpB, such as UhpB151::4. The presence of extracellular Glu6P could be signaled by the production of some effector molecule by UhpC or by a coupled conformation change in a UhpB-UhpC complex. Perhaps the binding of Glu6P to UhpC elicits a change in its conformation which alters the conformation of the transmembrane portion of UhpB by the direct contact of UhpB and UhpC in a complex in the cytoplasmic membrane. In the absence of inducer, the kinase portion of UhpB could be inhibited through its interaction with the transmembrane portion. Distortion of the conformation of the extramembranous loops by the direct coupling to UhpC with bound Glu6P might relieve this autoinhibition of UhpB function. UhpC alone cannot provide the site for inhibition of UhpB activity, because the loss of UhpC does not result in constitutive expression. It remains to be shown why some constitutive UhpB mutants require UhpC function and others do not. Explanations of this response will require measurements of the levels of the Uhp regulatory proteins in the mutants and analysis of the effect of altered protein stoichiometries. Whatever the mechanism, however, the interplay of the mutants in UhpB and UhpC indicates that signal transduction involves the physical interaction of these membrane-associated proteins.

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