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MalFGK Complex Assembly and Transport and Regulatory Characteristics of MalK Insertion Mutants

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MalK is a peripheral cytoplasmic membrane protein that has multiple activities in *Escherichia coli*. It associates with integral cytoplasmic membrane proteins MalF and MalG to form the maltose transport complex (MalFGK), a member of the ATP-binding cassette (ABC) superfamily of proteins. In addition, MalK participates in two different regulatory pathways which modulate *mal* gene expression and MalFGK transport activity. We have created a set of *malK* mutations for analysis of the protein's structure and folding. These mutations, distributed throughout *malK*, are all similar insertions of 31 codons. The ability of each mutant to function in maltose transport and MalK-dependent regulation was characterized. Furthermore, we have exploited a sensitive biochemical assay to classify our MalK insertion mutants into two additional categories: MalFGK complex assembly proficient and complex assembly defective. The regions containing the insertions in the assembly-proficient class should correspond to areas within MalK that are surface exposed within the MalFGK complex. Affected regions in assembly-deficient mutants may be involved in critical structural contacts within the complex. One mutant apparently blocks assembly at an intermediate stage prior to oligomerization of the final MalFGK complex. This work contributes to the analysis of ABC transport proteins and to the study of the assembly process for hetero-oligomeric membrane proteins.

Maltose transport across the cytoplasmic membrane of *Escherichia coli* utilizes a periplasmic binding protein-dependent transporter which is a member of the ATP-binding cassette (ABC or traffic ATPase) superfamily of proteins (reviewed in references 2, 8, and 14). In addition to the prokaryotic binding protein-dependent transporters, the ABC superfamily also includes several medically important eukaryotic proteins, including mammalian P-glycoprotein of multidrug-resistant tumor cells and the cystic fibrosis transmembrane conductance regulator. The maltose permease is a heterotetrameric complex comprised of integral membrane proteins MalF and MalG, which associate with peripheral membrane protein MalK in the stochiometry $MalFGK_2$ (4). Our goal is to characterize the assembly and folding of the $MalFGK_2$ complex to better understand the process of tertiary and quaternary folding of membrane proteins and the structural and functional interactions within the complex. Knowledge gained by the study of maltose permease also should contribute to the structure-function analysis of the other ABC superfamily proteins.

Here, we describe the results of our mutagenesis of the gene coding for MalK, which is the domain of the maltose transporter with the most extensive homology to other ABC transporters. MalK has been shown to have several distinct functions in the cell. First, MalK contains a well-defined nucleotide-binding fold (Walker boxes A and B) that has been shown to be essential for the hydrolysis of ATP that energizes maltose transport (4, 6, 9, 23). Second, MalK contributes to the regulation of *mal* gene expression by an uncharacterized (and possibly indirect) interaction with the *mal* transcriptional activator, MalT (17). Finally, MalK interacts with unphosphorylated enzyme IIA^{glc} ($EIIA^{glc}$) of the phosphoenolpyruvate: sugar phosphotransferase system in a second distinct regulatory pathway known as inducer exclusion. This interaction downregulates the transport activity of the transport complex (5, 9).

Previously, regions of MalK involved in maltose transport and in regulation by MalT and $EIIA^{glc}$ have been examined by using a variety of point and deletion mutants (5, 9, 20). However, the three-dimensional structure of MalK is not known and its intra- and intermolecular interactions remain largely uncharacterized. We have created a library of 13 in-frame *malK* insertion mutations by using a new mutagenic strategy (12). These insertions introduce 31 mostly hydrophilic amino acids into the protein. We characterized the impact of the insertional mutations on the different phenotypes associated with MalK. In addition, we determined the abilities of the mutants to properly assemble with MalF and MalG into the transport complex in the cytoplasmic membrane. This analysis broadens our understanding of the structure of MalK, particularly as it pertains to interactions with other proteins in the cell.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The expression of the *malK* alleles on pBT100 and its derivatives is under the control of the *trc* promoter and can be induced with isopropyl-β-D-thiogalactopyranoside (IPTG).

Media. The rich (LB), minimal (M63), and MacConkey media used have been described previously (13). Sucrose was added to plates at a final concentration of 5%. Other sugars were added to 0.2% or 1% for minimal or MacConkey medium. Antibiotics were added at final concentrations of 100 μg/ml for ampicillin (AMP), 15 μg/ml for tetracycline, and 30 μg/ml for chloramphenicol (CM) and kanamycin (KAN). 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) was added at a final concentration of 40 μg/ml. IPTG was added to a final concentration of 1 mM, except where noted otherwise.

General techniques. Standard techniques, such as plasmid preparation, cloning, transformation, and restriction endonuclease analysis, were used as previously described (19).

Construction of pBT100. The mutagenesis protocol (see below) works most easily when the target gene is carried on a plasmid with no *Bam*HI sites. To construct an appropriate *malK* expression plasmid, pMR11 (9) was digested with *Nco*I and *Hin*dIII. The *malK*-containing fragment was cloned into the *Nco*I/*Hin*dIII site of the vector p*Trc*99A. The resulting plasmid, called p99K8,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
Strains		
CC191	*recA1* Δ*lacX74 galE galK* F_{128} *lacI*^{q} *lacZ* Δ*M15*	10
MC4100	F$^-$ *araD* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 flbB5301 rbsR*	3
BT8	MC4100 *malT*(Con)	22
HS3169	MC4100 *malT*(Con) Δ*malK16 zjb-729*::Tn*10*	16
HS3628	*ptsH315* Δ*malK16 zjb-729*::Tn*10*	9
Plasmids		
p*Trc*99A	Derivative of pKK233-2 with *bla*, *lacI*^{q}, and *trc* promoter	1
pMR11	pACYC184 with *malK*	17
pBT100	p*Trc*99A with *malK*	This study
pLKZ16/pLK16	pBT100 Φ(*malK16′ lacZ*)/pBT100 *malK550*::*i31*	This study
pLKZ238/pLK238	pBT100 Φ(*malK238′ lacZ*)/pBT100 *malK551*::*i31*	This study
pLKZ268/pLK268	pBT100 Φ(*malK268′ lacZ*)/pBT100 *malK552*::*i31*	This study
pLKZ412/pLK412	pBT100 Φ(*malK412′ lacZ*)/pBT100 *malK553*::*i31*	This study
pLKZ442/pLK442	pBT100 Φ(*malK442′ lacZ*)/pBT100 *malK554*::*i31*	This study
pLKZ502/pLK502	pBT100 Φ(*malK502′ lacZ*)/pBT100 *malK555*::*i31*	This study
pLKZ634/pLK634	pBT100 Φ(*malK634′ lacZ*)/pBT100 *malK556*::*i31*	This study
pLKZ736/pLK736	pBT100 Φ(*malK736′ lacZ*)/pBT100 *malK557*::*i31*	This study
pLKZ826/pLK826	pBT100 Φ(*malK826′ lacZ*)/pBT100 *malK558*::*i31*	This study
pLKZ874/pLK874	pBT100 Φ(*malK874′ lacZ*)/pBT100 *malK559*::*i31*	This study
pLKZ1039/pLK1039	pBT100 Φ(*malK1039′ lacZ*)/pBT100 *malK560*::*i31*	This study
pLKZ1084/pLK1084	pBT100 Φ(*malK1084′ lacZ*)/pBT100 *malK561*::*i31*	This study
pLKZ1093/pLK1093	pBT100 Φ(*malK1093′ lacZ*)/pBT100 *malK562*::*i31*	This study

has a unique *Bam*HI site located downstream of *malK*. This site was removed to create pBT100 by digesting p99K8 with *Bam*HI, filling in the sticky ends with T4 DNA polymerase and religating with T4 DNA ligase.

Transposon mutagenesis. Mutagenesis of *malK* in strain CC191 containing pBT100 was carried out by using transposon Tn*lacZ*/in by adaptation of the procedure of Manoil and coworkers (11, 12). A replication-defective λ phage carrying the Tn*lacZ*/in transposon was added to cells at a multiplicity of infection of approximately 0.5. Each infection mixture was then divided into aliquots that were plated separately on LB (no NaCl) with AMP, CM, and sucrose and incubated at 30°C for 36 to 40 h. Cells were harvested from each plate, and plasmid DNA was isolated. This DNA was transformed back into CC191 (*lacZ*), and Tn*lacZ*/in insertions in pBT100 were selected by plating onto LB (no NaCl) with AMP, CM, sucrose, and X-Gal. Two alternative transposition products can be isolated by this procedure: (i) plasmids containing the entire Tn*lacZ*/in transposon (which specifies both CM and KAN resistance and sucrose sensitivity) and (ii) plasmids containing only the IS*lacZ*/in element (which only codes for CM resistance). Our selection conditions greatly favor the recovery of derivatives containing just the IS*lacZ*/in portion of the transposon (12). Blue colonies were picked and screened for KAN sensitivity (to ensure the presence of only the IS*lacZ*/in portion). Plasmid DNA was restriction mapped to determine the approximate locations of the insertion sites. Fusion junctions for insertions mapping in or near *malK* were sequenced with Sequenase, version 2.0 (U.S. Biochemical), on double-stranded templates. The DNA sequencing primer used (5′CGGGATCCCCCTGGATGG3′) hybridizes ca. 50 bases upstream of a fusion junction within the IS*lacZ*/in sequences.

After identification of the in-frame *malK-lacZ* fusions, the final mutagenesis step was carried out in vitro. Complete *Bam*HI digestion of the IS*lacZ*/in fusion plasmid, followed by religation, results in deletion of all but 93 bp derived from insertion sequences and 9 duplicated target gene bp (12). (The deleted sequences include the CM resistance gene of IS*lacZ*/in.) Cells were transformed with the digested and religated plasmids. AMP-resistant transformants were screened for the desired deletions by verifying their CM-sensitive and LacZ$^-$ phenotypes. A 31-codon insertion mutation occurs at the same location in the gene as the originally isolated gene fusion junction. It codes for the following sequence, given in the one-letter amino acid code: (S, P, T, or A)DSYTQVASWTEPFPFSIQG DPRSDQET(V, A, D, E, or G)XX, where the X residues are specified by the two codons 5′ to insertion sites that are duplicated during the transposition event.

Transport and regulation phenotypes of insertion mutants. The transport phenotypes of the MalK mutants were assayed in HS3169 transformed with the plasmids expressing the various *malK* alleles. This *malT*(Con) strain has constitutive expression of the *malEFG* genes, which are also required for maltose transport. The Mal phenotype of these strains was tested on maltose-minimal–AMP and maltose-MacConkey agar-AMP plates (without IPTG).

Maltose uptake was quantified in plasmid-containing HS3169 derivatives grown at 37°C (without IPTG induction) in LB-AMP to an A_{600} of 0.5. Cells were washed and resuspended in M63 salts, which served as the buffer for the assays. [^{14}C]maltose (Amersham Corp.; specific activity, 591 mCi/mmol) was present at 0.1 μM, and the total maltose concentration was 1.1 μM. Sixty-microliter portions were removed from a total assay volume of 240 μl at 1, 2, 3, and 5 min and collected on 0.45-μm-pore-size HA Millipore filters. Filters were washed with 5 ml of 0.5 M LiCl, dried, and counted in scintillation vials with Aquasol (Du Pont-NEN). Results are presented as the average uptake per minute in assays that were approximately linear over 5 min. Each sample was corrected for the counts present in control HS3169 assays.

The abilities of the MalK mutants to participate in MalT-dependent *mal* regulation and EIIA^{glc} regulation of maltose transport activity were tested in strains transformed with the various plasmid constructs (9, 17). *mal* gene regulation was assayed in *malT*$^+$ strain MC4100 on maltose-MacConkey agar-AMP plates with or without added 1 mM IPTG. The ability of the MalK mutants to interact with EIIA^{glc} was tested in PtsH$^-$ strain HS3628 (9) on maltose-MacConkey agar-AMP plates with or without 0.1% α-methylglucoside (α-MG).

Proteolysis test of MalFGK complex assembly. The assembly state of the MalFGK proteins in the cell was measured by assaying the trypsin sensitivity of the MalF protein in spheroplasts as previously described (22). HS3169 cultures containing the different *malK* insertion mutation plasmids were grown in M63 medium (with glycerol, AMP, and all amino acids except cysteine and methionine) at 37°C to an A_{600} of 0.2. Expression of *malK* in the different strains was induced with IPTG for 60 min prior to conversion of the cells to spheroplasts. Proteolysis was carried out with 25 μg of trypsin per ml at 0°C for 20 min. Protease treatment was terminated by addition of phenylmethylsulfonyl fluoride (PMSF), and spheroplasts were harvested and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. The MalF protein in the cell extracts was detected by Western blot analysis.

Western blots and antibodies. Western blots were developed as previously described (22), after transfer of proteins from sodium dodecyl sulfate–12.5% polyacrylamide gels onto 0.2-μm-pore-size nitrocellulose. Primary antibodies were specific for β-galactosidase (from 5 Prime→3 Prime, Inc.), for MalF (22), for MalK (21), or for the insertion created by mutagenesis (12). The secondary antibody used was goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Boehringer Mannheim).

Chemicals and enzymes. IPTG and X-Gal were purchased from Bachem. Maltose was obtained from Pfanstiehl Laboratories. Other medium supplies were obtained from Difco, Fisher Scientific, or Sigma. Lysozyme, PMSF, and α-MG were purchased from Sigma. Trypsin was obtained from Worthington. Restriction and DNA modification enzymes were from New England Biolabs.

RESULTS

Isolation of *malK-lacZ* fusions and *malK* insertion mutations. To obtain insertion mutations in *malK*, two-step Tn*lacZ*/in transposon mutagenesis was carried out on cells

containing *malK* plasmid pBT100. Isolation of in-frame insertion mutations requires that the IS*lacZ*/in portion of the transposon be inserted into the *malK* gene in only one orientation and in one of the three possible reading frames (12). In the first step, translational *malK-lacZ* fusions were isolated in vivo.

Cells expressing MalK-LacZ fusion mutants were identified among heterogeneous pools containing 12,000 plasmid-linked transposon mutant colonies by screening for blue colonies on media containing X-Gal. Of the 120 independent $LacZ^+$ mutants analyzed, 119 contained only the IS*lacZ*/in element. Plasmids were isolated from the $LacZ^+$ cells, and restriction enzyme analysis was used to determine the approximate locations of fusion junctions. Of these, a subset were characterized by DNA sequencing to determine the precise location of the IS*lacZ*/in insertion. We isolated 13 different in-frame fusion constructs in this mutagenesis (each on a separate pLKZ plasmid; Table 1). Each *malK-lacZ* fusion was given a name which designates the nucleotide immediately before the insertion. Five of the IS*lacZ*/in insertions were isolated twice (after *malK* bp 238, 268, 412, 442, and 736), and one was isolated three times (after bp 502).

The *malK-lacZ* fusions were assayed for expression of stable fusion proteins via Western blot analysis with β-galactosidase antibodies and for complementation of *malK* mutant strain HS3169 for maltose transport on maltose-minimal and MacConkey medium plates. While all 13 MalK-LacZ fusions were detected as full-length proteins in the cell (data not shown), none functioned in maltose transport.

In the second step of the mutagenesis protocol, plasmids containing the 13 *malK-lacZ* fusion constructs were manipulated in vitro to remove most of the IS*lacZ*/in sequences. Each 31-codon insertion mutation (carried on pLK plasmids) was assigned an allele number, as shown in Table 1.

Mal phenotypes of insertion mutants. The MalK mutants were tested for the ability to participate in maltose transport in HS3169 on maltose-minimal (data not shown) and maltose-MacConkey agar (Table 2) plates. Seven *malK* alleles complemented the Δ*malK* strain for maltose transport on plates to various extents. Of the 13 insertion mutations, 6 displayed a Mal^- phenotype on both minimal agar and MacConkey agar. Only one of the Mal^- alleles, *malK552*, was dominant with respect to $malK^+$ in related Mal^+ strain BT8 (Table 2).

We more precisely determined the relative maltose transport activities of the mutants with [^{14}C]maltose uptake assays (Table 2). The most active mutants (MalK550, MalK556, and MalK561) retained ca. 20 to 80% of the wild-type maltose uptake activity. Mutants that appeared inactive for transport in the plate assays ranged from 1.5 to 3.5% of wild-type activity. Surprisingly, two mutants (MalK559 and MalK560) that appeared somewhat active for transport on both maltose-minimal and MacConkey agar plates retained only 2.6 to 3.0% of the wild-type [^{14}C]maltose uptake activity, similar to some apparently Mal^- mutants. This discrepancy is presumably due to differences in the way transport was tested in the different assays.

Detection of mutant MalK proteins. A trivial explanation for the variation in the abilities of the different *malK* mutations to complement the Δ*malK* strain for maltose transport is that the different mutant proteins are present in widely varying levels in the cell. This could be due to differential sensitivity of the mutant proteins to endogenous intracellular proteases. To test this possibility, we examined the abundance of the MalK mutant proteins in the cell by Western blot analysis with an antiserum specific for the 31-residue insertion (Fig. 1). Five of six Mal^- mutants, and all of the Mal^+ mutants, showed significant and roughly equivalent amounts of MalK protein accumulation in their cells. Minor breakdown products were detected in a few extracts (e.g., for MalK558). We concluded that the Mal^- phenotype of most HS3169 derivatives is not attributable to instability of the mutant MalK proteins.

TABLE 2. Transport phenotypes of MalK mutants

MalK derivative	Growth on maltose-MacConkey agar[a]	Maltose uptake[b] (%)	Dominance[c]
MalK550	+++	17	NT
MalK551	−	3.0	No
MalK552	−	2.3	Yes
MalK553	−	2.3	No
MalK554	−	1.6	No
MalK555	−	3.5	No
MalK556	+++	78	NT
MalK557	−	2.2	No
MalK558	+	7.9	NT
MalK559	+	2.6	No
MalK560	+	3.0	No
MalK561	+++	41	NT
MalK562	++	11	NT
$MalK^+$	+++	100	NT

[a] This test was performed with strain HS3169 transformed with pBT100 or a pLK plasmid. Phenotypes were scored after growth at 37°C for 15 h. +++, dark red colony with bile salt precipitation; ++, dark pink colony with no salt precipitation; +, light pink colony; −, white colony.

[b] Uptake activity was measured as average counts per minute incorporated by 2.5×10^7 cells at 25°C, in comparison with that of HS3169 transformed with pBT100. 100% = 1,100 cpm.

[c] Dominance of the *malK*::*i31* mutations with respect to $malK^+$ was tested in BT8 transformed with a pLK plasmid, on maltose-MacConkey agar plates with 1 mM IPTG. Phenotypes were scored after growth at 37°C for 15 h. Yes, white colony, corresponding to a dominant mutation. No, red colony, corresponding to a recessive mutation. NT, not tested. All strains containing *malK*::*i31* plasmids were red on plates without IPTG.

No mutant protein was detected with the anti-insert antibody for MalK557. Therefore, we assayed for the presence of any stable MalK peptide in the cells. Such a peptide might carry out some functions of MalK in vivo. A Western analysis of mutant MalK557 was done with polyclonal antibodies directed against MalK, which have been shown to bind with MalK proteolytic peptides (21). Even in this analysis, we did not detect any MalK peptides derived from MalK557 (data not shown). At the same time, we compared the levels of several of the plasmid-encoded MalK variants in HS3169 to that of the wild-type protein expressed from a chromosomal copy of *malK*. The background, uninduced level of MalK in the plasmid-containing HS3169 derivatives was ca. 10 to 50% of that in

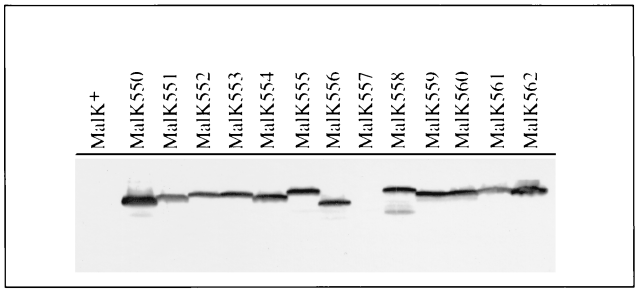

FIG. 1. MalK mutant proteins. Cell extracts were made from cultures grown in LB with AMP to an A_{600} of 0.2 and then induced with IPTG for 30 min. A 10-μg sample of total cellular protein was loaded per gel lane. Mutant proteins on the Western blot were detected with antipeptide antibodies directed against the 31-amino-acid insert. The induced levels of MalK expressed from plasmids after this induction with IPTG were ca. two to four times that of chromosomally expressed MalK in BT8 (data not shown).

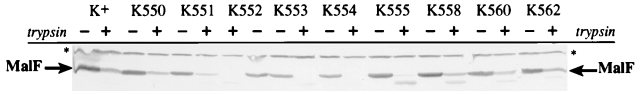

FIG. 2. Ability of MalK mutants to form a MalFGK complex. This representative Western blot shows the ability of MalK variants to assemble a MalFGK complex in which MalF is resistant to trypsin cleavage. Cultures were induced with IPTG so that MalK levels in the cell would not be limiting for MalFGK complex assembly. The position of intact MalF is shown; a background band (present in a Δ*malF* strain) which is also recognized by the MalF antiserum is indicated by asterisks. −, no-protease controls; +, 25 μg of trypsin added. Beneath the intact MalF band in many trypsinized samples are one or two large MalF proteolytic peptides produced to various extents in different repetitions of this experiment for all strains, including the MalK$^+$ control.

related Mal$^+$ strain BT8, where *mal* expression is constitutive (data not shown).

Assembly of mutant MalK proteins into the MalFGK complex. For the MalK mutants that make stable proteins, we reasoned that there are two plausible explanations for the variation in the ability to participate in maltose transport. One is that the insertion mutation might disrupt the protein's ability to hydrolyze ATP or to couple the energy of hydrolysis with maltose transport. A second possibility is that the 31-residue insertion might interfere with interactions between MalK and the integral membrane proteins of the transport complex, MalF and MalG.

Previously, we described an assay for the assembly of the hetero-oligomeric MalFGK complex based on the proteolytic sensitivity of MalF (22). Briefly, periplasmic domains of MalF vary in sensitivity to exogenous proteases, such as trypsin or chymotrypsin, depending on whether the MalF protein is assembled into the MalFGK complex. If MalF is not stably associated with both MalK and MalG, it is highly sensitive to proteolytic cleavage of its periplasmic domains in spheroplasted cells. If the complex is formed correctly, MalF is protease resistant.

To determine whether the transport-defective MalK mutants had lost the ability to form a stable MalFGK complex, proteolysis experiments were performed. If a MalK mutant was competent for MalFGK complex assembly, the MalF present in the cell should be largely resistant to proteolytic degradation by trypsin. Conversely, if a MalK mutant had lost its ability to stably assemble with MalF and MalG, the MalF present in the cell should be highly sensitive to trypsin. Spheroplast preparations were made from HS3169 cells expressing the different MalK mutants. No-protease control and trypsin-treated samples were analyzed by use of Western blots developed with MalF-specific antibodies (Fig. 2 and data not shown).

All of the MalK mutants proficient in maltose transport were able to support a high level of MalFGK complex assembly, as evidenced by the substantial level of protease-resistant MalF present in the spheroplast preparations. Among the transport-defective MalK mutants, MalK551 and MalK555 were also able to assemble the MalFGK complex. In contrast, the MalK552, MalK553, and MalK554 mutants were highly defective in complex assembly.

Some MalK mutants lose the ability to negatively regulate the maltose regulon. Shuman and coworkers have shown that overproduction of MalK in wild-type Mal$^+$ cells results in repression of the maltose regulon (9, 17). This regulation is due to an uncharacterized interaction between MalK and MalT. Normally, *malT*$^+$ *malF*$^+$ *G*$^+$ *K*$^+$ strains (such as MC4100) expressing high levels of MalK display a Mal$^-$ phenotype. [This phenotype is not observed in a *malT*(Con) strain such as BT8.] We tested whether our MalK insertion mutants could participate in this MalT-dependent regulation.

TABLE 3. MalK-dependent *mal* regulation[a]

MalK derivative	Mal phenotype	
	Without IPTG	With IPTG
MalK550	+	−
MalK551	+	−
MalK552	+	−
MalK553	+	−
MalK554	+	−
MalK555	+	−
MalK556	+	−
MalK557	+	+
MalK558	+	+
MalK559	+	+
MalK560	+	+
MalK561	+	−
MalK562	+	+
MalK$^+$	+	−
No MalK	+	+

[a] This test was performed with strain MC4100 transformed with a pLK plasmid, pBT100 (MalK$^+$), or p*Trc*99A (no MalK). +, red colony with bile salt precipitation; −, white colony.

The 13 *malK* mutation plasmids in Mal$^+$ strain MC4100 were plated on maltose MacConkey agar with and without IPTG (Table 3). All of the transformants exhibited a Mal$^+$ phenotype in the absence of IPTG. Strains with five of the *malK* mutation plasmids showed a Mal$^+$ phenotype in the presence of IPTG. One of these was the plasmid containing the *malK557* allele. As this mutation does not allow expression of a stable protein, it should not repress the *mal* regulon under any conditions. The other four MalK mutants, with insertions after residues 275, 291, 346, and 364, apparently have lost the ability to participate in MalT-dependent *mal* gene regulation. The remaining mutants were Mal$^-$ in the presence of IPTG, indicating that they retain this ability. This latter group includes MalK561, whose mutation at codon 361 maps between others that are insensitive to this regulation.

We also tested the participation of the *malK-lacZ* fusion constructs in this regulation in strain MC4100. Strains containing these plasmids were Mal$^+$ on maltose-MacConkey agar plates with and without IPTG. This indicates that these fusion proteins cannot function in *mal* regulation. Furthermore, the Mal$^-$ *malK-lacZ* fusions are fully recessive with respect to *malK*$^+$.

EIIA^{glc} interaction with MalK mutants. Maltose transport is inhibited ca. 70% in cells containing high levels of unphosphorylated (Pi$^-$) EIIA^{glc} (5, 18). In vivo, this elevated level of Pi$^-$ EIIA^{glc} can be found in cells with a defect in early steps of the phosphoenolpyruvate:sugar phosphotransferase system cascade (such as a PtsH$^-$ mutant) growing in the presence of α-MG, a nonmetabolizable glucose analog. Some *malK* mutations relieve this inhibition and can restore maltose transport to high levels in the presence of α-MG (5, 9).

We tested whether our MalK mutants that were active in maltose transport were susceptible to EIIA^{glc} regulation. HS3628 strains containing the appropriate plasmids were screened in a plate assay for maltose transport with and without α-MG (Table 4). The expression of some MalK mutants had to be increased with submaximal inducing levels of IPTG to complement this Δ*malK* strain for maltose transport. Even with the induction of *malK*::*i31* expression, the two lowest-

TABLE 4. MalK interaction with $EIIA^{glc}$

MalK derivative (IPTG concn [μm])	Mal phenotype[a] on:	
	Maltose-MacConkey agar	Maltose MacConkey agar + αMG
$MalK^+$	+++	−
MalK550	+++	−
MalK556	+++	+
MalK556 (10)	+++	++
MalK558	−	−
MalK558 (10)	++	−
MalK559	−	−
MalK559 (10)	−	−
MalK559 (100)	−	−
MalK560	−	−
MalK560 (10)	−	−
MalK560 (100)	−	−
MalK561	−	−
MalK561 (10)	++	−
MalK562	+	+
MalK562 (10)	+++	++

[a] Colony phenotypes were scored as described in Table 2, footnote *a*.

activity MalK mutants did not support maltose transport in HS3628. MalK556 and MalK562 were still Mal^+ in the presence of α-MG and appeared resistant to $EIIA^{glc}$ inhibition. Three other mutants retained wild-type sensitivity to $EIIA^{glc}$-mediated repression of transport.

DISCUSSION

We have carried out a mutational analysis of the *malK* gene to gain a greater understanding of the protein's intra- and intermolecular interactions. The locations of the *malK*::*i31* mutations isolated and a summary of their phenotypes are shown in Fig. 3. MalK derivatives that were compromised in transport or regulation generally followed a pattern suggested in other reports (5, 9, 20): most mutants with an insert in the N-terminal portion of the protein were severely defective in maltose transport; mutants defective in MalT and/or $EIIA^{glc}$ regulation had inserts in the C-terminal half of the protein. The exceptions to these generalizations provide new structural clues

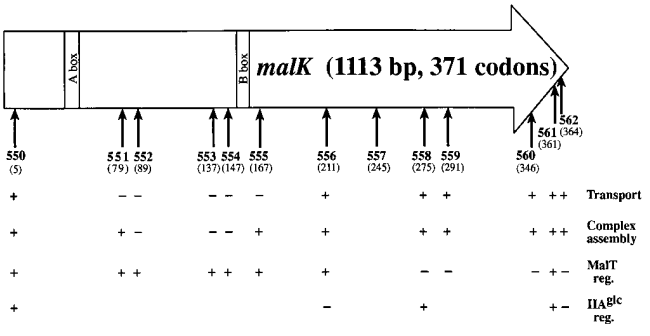

FIG. 3. Summary of *malK* mutations. The locations of the *malK*::*i31* mutations isolated in this study are indicated by vertical arrows. The allele number for each mutation is given beneath each arrow; the codon immediately preceding each insertion is in parentheses. The positions of the consensus Walker A and B boxes for nucleotide binding (as shown in reference 2) also are indicated. The phenotypes of the mutants for complementation of maltose transport (Transport), association with MalF and MalG (Complex assembly), MalT-MalK-dependent *mal* gene regulation (MalT reg.), and $EIIA^{glc}$-dependent inhibition of maltose transport (IIA^{glc} reg.) are summarized at the bottom. +, $MalK^+$ phenotype, −, $MalK^-$ phenotype. No phenotypes are given for *malK557*, as this mutation does not allow expression of a stable protein in the cell.

about MalK. In addition, our ability to separate the Mal^- MalK mutants defective in complex assembly from those proficient in assembly provides information about the roles that different regions of MalK play during oligomerization of the MalFGK complex. These mutant proteins will help us analyze the folding process and the ultimate conformation of MalFGK and will provide insights into these aspects of other ABC superfamily proteins.

We identified five different MalK mutants (MalK551, MalK552, MalK553, MalK554, and MalK555) that were deficient in maltose transport in all tests yet still make significant amounts of stable protein in the cell (Table 2 and Fig. 1). While the Mal phenotypes of these mutants were similar, their behavior in MalFGK complex assembly was quite different.

The MalK551 and MalK555 mutants were able to assemble with MalF and MalG, as demonstrated by the MalF proteolysis assay (Fig. 2). However, these mutations are recessive with respect to $malK^+$ (Table 2). These insertions lie at the beginning (MalK551) or the end (MalK555) of a region that has been proposed to be important for transport activity and/or complex assembly (8, 14). The phenotypes of these two mutants suggest that the N and C termini of this region may, in fact, be important for transport activity but not crucial for complex assembly. The lack of dominance of these mutations could stem from a higher affinity of wild-type MalK for MalF and MalG in the transport complex in a $malK^+$/*malK* merodiploid cell. The $MalK^+$ protein may simply displace the mutated version from the complex. We consider this particularly plausible in regard to the MalK555 protein, which has a reproducible but limited ability to support MalFGK complex assembly.

We can suggest an alternative explanation for the phenotypes of the recessive *malK551*::*i31* mutation. Walter et al. have isolated MalK mutants with an amino acid substitution at residue 82 which are still active as ATPases but have somewhat reduced maltose transport activity (23). The Mal^- MalK551 protein may be active as an ATPase (as the insertion occurs between the Walker A and B boxes, after residue 79; Fig. 3); however, the protein might not undergo the conformational shifts required during substrate transport. This defect might be compensated for by $MalK^+$ in a $MalFGK^+K551$ complex.

Two other recessive mutations, *malK553* and *malK554*, express proteins that do not assemble into the $MalFGK_2$ complex. The inserts of these mutants are immediately N terminal to the Walker B site of MalK, which is essential for the hydrolysis of ATP that is coupled to substrate transport (Fig. 3) (4, 6, 9). The lack of complex assembly seen with these mutants suggests that the area of MalK around and including the B box is critical for MalK-MalFG interactions.

MalK552, whose insertion is only 10 residues more C terminal than that of MalK551, shows a distinct pattern in its associations with MalF and MalG. This mutant is unable to assemble into the final $MalFGK_2$ complex (Fig. 2). Furthermore, the *malK552* mutation is dominant with respect to $malK^+$ (Table 2). We propose that this mutant protein is able to form a heterodimer or -trimer en route to the final complex but a subsequent step in assembly is blocked by the insert lesion. That is, this mutant is proficient in the formation of only a subset of interactions with MalF, MalG, or MalK. Further study of this mutant should be valuable in the determination of the assembly pathway for the maltose transport complex.

Previously, Panagiotidis et al. used cellular fractionation assays to examine the association of different MalK mutants with MalF and MalG (16). They characterized dominant Mal^- missense mutations in the regions of *malK* that code for the protein's Walker A and B boxes. The mutant proteins were defi-

cient in ATP binding or hydrolysis, but they did not differ detectably from wild-type MalK in their association with MalFG. In contrast, some of our Mal$^-$ mutations, found throughout the promoter-proximal portion of *malK*, were defective in the ability to interact with MalF and MalG. Our isolation of more severe insertion mutations rather than less disruptive amino acid substitutions may have enabled us to identify different structural features of MalK, particularly in regard to complex assembly. We characterized two distinct classes of assembly-defective MalK mutants. One of these is novel, containing the MalK551 mutant, which inhibits MalFGK$^+$ assembly. An added benefit of our analysis is that the locations of the insertions in the complex assembly-proficient MalK551 and MalK555 mutants may indicate regions that are tolerant of a substantial insertion when complexes are oligomerized. These may correlate with surface-exposed regions of MalK within the MalFGK complex. This proposal is consistent with our observations of 31-residue *lac* repressor insertion mutants (also created via Tn*lacZ*/in mutagenesis; 15). In that analysis, fully and partially active LacI mutants contained insertions in regions corresponding to those that were surface exposed in the crystal structure of the wild-type tetrameric repressor.

The final mutation that causes a Mal$^-$ phenotype, *malK557*, fails to express a mutant protein that accumulates to detectable levels in the cell (Fig. 1). This suggests that MalK cannot tolerate a large hydrophilic insertion around residue 246. We suspect that this region of MalK is not normally surface exposed and that the insertion renders the MalK557 protein so unstable that it is completely degraded by intracellular proteases. The frequency of such destabilizing mutations isolated in *malK* (1 in 13) by this mutagenic strategy is similar to that observed for LacI (2 in 18) (15).

The other seven *malK*::*i31* mutations all were able to complement a *malK* deletion for transport, although some displayed a phenotype more closely resembling that of the wild type than others (Table 2). Two mutants, MalK550 and MalK561, retain all of the functions of the wild-type protein: complex assembly, transport, MalT-dependent regulation, and EIIA^{glc} regulation (Fig. 3). The MalK550 insertion is after Gln5, suggesting that the N terminus of MalK is surface exposed within the MalFGK complex. The ability of MalK550 to participate in maltose transport is in contrast to the Mal$^-$ MalKΔSer3Val4 isolated by Walter et al. (23). While the ability of MalK550 to function in MalT- and EIIA^{glc}-dependent regulation is not surprising, it is notable for the MalK561 mutant. It is clear that other insertions mapping near the *malK561* mutation impair the mutant protein's function in one or both of these regulatory pathways. We suggest that the region around Ala361 is surface exposed and that the insertion in MalK561 is oriented away from protein-protein contact sites required for these activities.

The MalK556 and MalK558 proteins were both proficient in all but one of the functions of wild-type MalK (Fig. 3). MalK556 is the insertion mutant with the highest maltose transport activity (Table 2), but it is immune to EIIA^{glc}-dependent repression of maltose transport. In contrast, MalK558 is not recognized by the factor required for MalT-dependent repression of *mal* expression. Point mutants with similar phenotypes have previously been identified (5, 9), but it is notable that a large insertion mutant could have a phenotype similar to that of an amino acid substitution mutant.

The *malK562* mutation has its insertion only three codons downstream of *malK561* (Fig. 3). This alteration of the insertion site may cause loss of both MalT- and EIIA^{glc}-dependent regulation in the MalK562 mutant. That is, the insertion may prevent the protein contacts necessary for these regulatory events. Alternatively, the variation in the insertion sequence between the two mutants may lead to the difference in the regulatory phenotypes of the two proteins. (For MalK561, the first residue in the insertion lesion is Ser; for MalK562, the first residue is Pro.)

The MalK559 and MalK560 mutants behaved similarly in all phenotypic tests (Table 2 and Fig. 3). They displayed very low [^{14}C]maltose uptake activity, were deficient in MalT-dependent regulation, and could not be tested for EIIA^{glc}-dependent regulation. The transport phenotypes of these mutants were measured in uninduced cells. The steady-state level of these mutant proteins was lower under uninduced conditions than those of most other mutants (data not shown). We believe that the low abundance of these proteins contributes to the poor performance of these mutations in the transport activity tests. However, we cannot easily explain our inability to test the EIIA^{glc}-dependent regulation of these mutants.

We did not detect any significant difference among the transport-proficient MalK mutants, including MalK559 and MalK560, to support complex assembly (as measured by the MalF proteolysis assay, done with IPTG-induced cells), regardless of their respective transport activities. As most of these mutations map in the promoter-distal half of the gene, one might conclude from this that there are no essential interactions between the C-terminal portion of MalK and integral membrane proteins MalF and MalG. In contrast, deletion mutant studies by Schneider and Walter (20) suggest that residues 293 to 317 of MalK are critical for interaction with MalF and MalG. The fact that none of our *malK-lacZ* fusions (expressing C-terminally truncated MalK derivatives) was able to complement a *malK* deletion for transport also is consistent with some requirement of the C-terminal region of MalK for maltose transport. Alternatively, the Mal$^-$ phenotype of the MalK-LacZ fusions might be due to steric interference from the folding and oligomerization of the LacZ moiety of the fusion protein. However, this ability to interfere is not absolute. A large, three-part MalK-LamB-LacZ hybrid protein can participate in maltose transport (7), possibly dependent on the lengthy LamB linker region for MalK activity. The exact nature of any MalFGK interactions occurring within the C terminus of MalK is still unclear and warrants further investigation.

Our results have confirmed and expanded those from previous studies showing the association of different functions of MalK with different regions of the protein. We also present important new evidence concerning structural aspects of MalK during MalFGK assembly and within the final complex. Disruptions in the region around MalK residues 137 to 147 cause an inability of MalK to associate stably with MalF or MalG. In contrast, an insertion around MalK residue 89 produces a mutant protein that apparently supports some, but not all, of the steps of MalFGK$_2$ complex assembly. Finally, insertions around MalK residues 79 and 167 allow assembly of inactive MalFGK complexes. These mutant proteins should help us analyze the folding of hetero-oligomeric proteins and provide insights into this process for other ABC superfamily proteins.

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