MalK, the ABC component of the *Escherichia coli* maltodextrin transporter, inhibits the transcriptional activator MalT by antagonizing inducer binding

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SUMMARY

MalK, the ATP-binding cassette (ABC) component of the *Escherichia coli* maltodextrin transporter, has long been known to control negatively the activity of MalT, a transcriptional activator dedicated to the maltose regulon. By using a biochemical approach and the soluble form of MalK as a model substrate, we demonstrate that MalK alone inhibits transcription activation by MalT in a purified transcription system. The inhibitory effect observed in vitro is relieved by maltotriose, and by two *malT* mutations and one *malK* mutation known to interfere with MalT repression by MalK in vivo. MalK interacts directly with the activator in the absence of maltotriose, but not in the presence of maltotriose. Conversely, MalK inhibits maltotriose binding by MalT. Altogether, these data strongly suggest that MalK and maltotriose compete for MalT binding. Part, if not all, of the MalK-binding site is located on DT1, the N-terminal domain of MalT. All of these features indicate that MalK inhibits MalT by the same mechanism as two other proteins, MalY and Aes, that also act as negative effectors of MalT by antagonizing maltotriose binding by MalT. These results offer new insights into the mechanism by which gene regulation can be accomplished by the ATPase component of a bacterial ABC type importer.
INTRODUCTION

Recent studies revealed that bacterial transport systems can be directly involved in signal-transduction pathways and play a key role in gene regulation by displaying a regulatory function that is tightly coupled to their transport activity. Based on the few examples of transport-based sensory systems so far characterised in detail, the mechanisms of signal transduction differ widely, depending on the system. For instance, BglF, a phosphotransferase system (PTS) enzyme II that catalyses the import and phosphorylation of β-glucosides, phosphorylates and sequesters the BglG antiterminator in the absence of β-glucoside transport, and dephosphorylates and releases it when transport resumes (1). Active sugar transport triggers the recruitment of the Mlc repressor by the dephosphorylated glucose-specific PTS enzyme II (PtsG), thereby derepressing the Mlc-controlled genes (for a review, see (2)). In the case of the ferric-citrate transport system, substrate binding to the receptor located in the outer membrane triggers the activation of a specific sigma factor via a complex signalling cascade (3).

This paper deals with the roles that ATP-binding cassette (ABC) transporters play in signalling pathways. The model system is the E. coli maltodextrin transporter whose ABC component, the MalK protein, down-regulates the activity of MalT, a transcriptional activator that controls the expression of the maltose regulon (4). MalT control by MalK was first recognized with the finding that malK null mutants express constitutively the maltose regulon, whereas MalK overproduction blocks mal gene induction (5, 6). The observation that repression by MalK is relieved by both MalT overexpression and a class of malT mutations (malT') conferring constitutive expression of the maltose regulon further suggested that MalT is the target of the negative control exerted by MalK (6).

The maltodextrin transport system is one of the best characterised ABC transporters and its study has provided a wealth of information about the mechanism of
coupling between transport and ATP hydrolysis (7). The physiology of the regulatory function of MalK is also well understood. MalT repression by MalK ensures that the maltose regulon is not induced by endogenous maltotriose – the inducer of the system – in the absence of external maltodextrins (8). The concentration of maltotriose of internal origin is indeed high enough to cause full expression of the regulon in a malK strain in minimal medium supplemented with glycerol (8). The uninduced level of expression of the maltose regulon observed in a wild-type strain in the absence of maltose in the growth medium corresponds to residual MalT activity that escapes repression by MalK. In contrast, the mechanism of MalT inhibition by MalK remains unclear. Some MalT variants are known to display a higher affinity for maltotriose and to be less sensitive to repression by MalK, which suggests that MalK inhibits MalT by competing with maltotriose (6, 9). However direct evidence for this model is still lacking. Böhm et al. (10) identified a surface determinant on the C-terminal domain of MalK that is specifically involved in MalT repression and might represent the MalT binding site. Panagiotidis et al. (11) observed a MalT/MalK interaction in vitro, but this MalT/MalK complex did not respond to signals known to relieve MalT repression by MalK in vivo. Also, how maltodextrin entrance is coupled to derepression remains elusive. On the basis of genetic data, Panagiotidis et al. (11) proposed that only the ATP-bound form of MalK, which is expected to predominate when the transporter is resting, is able to repress MalT.

MalT is the archetype of a family of ~100-kDa transcriptional activators found in prokaryotes (12, 13). Transcription activation by MalT involves MalT self-association, cooperative binding to an array of MalT sites located in the target promoters and stimulation of open complex formation by the RNA polymerase (14-16). A complex interplay of signalling compounds and proteins control the activity of the MalT protein (4). MalT is active only in the presence of ATP and maltotriose, both of which are required for multimerisation (16). In addition to MalK, two other proteins, MalY, a β C-S lyase and Aes, an acyl esterase down-regulate MalT activity (17, 18). Recent studies have
revealed that both MalY and Aes interact directly with MalT and compete with maltotriose for MalT binding (19, 20). Available data suggest that unliganded MalT is in an equilibrium between an inactive, monomeric form and an active, monomeric form, prone to self-association. The inactive form would be stabilised by MalY or Aes, whereas the active form would be stabilised by maltotriose. The requirement for ATP as a positive effector of MalT might reflect a role for the ATPase activity of MalT in the competition between positive and negative effectors. Indeed, both ATP and ADP can promote MalT multimerisation or inhibitory complex formation, but ATP is more effective in driving MalT self-association whereas ADP is more effective in promoting formation of an inhibitory complex (16, 19, 20).

Structural studies have revealed that MalT is composed of four domains (21). DT1 (residues 1-241) binds and hydrolyses ATP (21). It also binds MalY and Aes (20, 22) while maltotriose is bound by DT3 (residues 437-806) (21, 23). DT4 (residues 807-901), which belongs to the LuxR-type family of DNA-binding domains, contains the DNA-binding site and most likely also carries a surface determinant contacting the RNA polymerase (24). The transition between the inactive and the active form of MalT involves DT1, DT2 and DT3, three domains that are specific to the MalT family of transcriptional activators and are thought to constitute a signal integration module (21).

To gain insight into the mechanism whereby MalK modulates MalT activity, we developed an in vitro system that reproduces MalT inhibition by MalK. The observation that MalK causes a Mal− phenotype when overproduced (6) suggested that free MalK, i.e. the MalK form that is not associated with the membrane components of the transporter (MalF and MalG), is able to inhibit MalT. We therefore used the soluble form of MalK as a model substrate. We show that MalK alone impedes transcription activation by MalT in a purified transcription system. Characterisation of the repression process revealed that MalK inhibits MalT by forming a complex with the activator and blocking inducer binding. Part, if not all, of the MalK-binding site is present on DT1.
EXPERIMENTAL PROCEDURES

Strains and plasmids

E. coli BL21(λDE3) has been described by Studier et al. (25). BL21(λDE3) ΔmalT220 contains a deletion of the entire malT gene (19). Plasmids pOM163 and pOM163-T38R are pET28b(+) (Novagen) derivatives producing DT1 or DT1-T38R with a Strep tag at their C-terminus (DT1S) (20). Plasmid pOM174 was obtained by amplifying the MalK encoding fragment of pKN101 (obtained from K. Nikaïdo) by PCR using oligonucleotides KU001 (5' - GCGGC CGCCATGGGGACCACGCATCAGGTCGA) and KD001 (5' - GGCGCG CAAAGCTTCAGGCTTTGTGTTTGT), digesting it with NcoI and HindIII, inserting it between the NcoI and HindIII sites of pET28b(+), followed by the insertion of a His-tag encoding linker, HTAG1/HTAG2 (HTAG1, 5' - CATGCATCATCACCATCACCATCA; HTAG2, 5' - CATGTGATGGTGATGATGATGATG) in the NcoI site of the intermediate construct. The sequence of the construct was verified. The encoded polypeptide has a M(H)6MG extension at the MalK N-terminus. The pOM174-G346S derivative was obtained by replacing the EcoRI-HindIII fragment of pOM174 by the EcoRI-HindIII fragment of pAB204 (10).

Chemicals

ADP containing <0.2% ATP was purchased from Roche Molecular Biochemicals. [14C]maltotriose (900 mCi/mmol) was obtained from America Radiolabeled Chemicals.

Proteins

RNA polymerase holoenzyme (Eσ70) was from Epicentre. Wild-type MalT, MalT^26 and MalT T38R proteins were purified in the presence of ATP as described by Danot and Raibaud (15), Schreiber et al. (19) and Joly et al. (20), respectively. ATP-free MalT was prepared by precipitating the purified protein with ammonium sulfate and filtering the
resuspended material through a G-75 Sephadex column according to Schreiber and Richet (16). The concentration of MalT was measured as described (16). ATP-free MalT was used throughout this work.

The MalK protein, with a His-tag at its amino terminus, was purified from strain BL21(λDE3) harboring pOM174. Bacteria were grown at 37°C in 1.5 liters of LB medium (5 g yeast extract, 10 g tryptone, 10 g NaCl, adjusted to pH 7) in the presence of 50 µg/ml kanamycin, induced with 1 mM isopropyl thio-β-D-galactoside (IPTG) at $A_{600} = 0.8$, and further grown at 18°C for 3 hours. Cells were harvested by centrifugation, resuspended in buffer A (20 mM Tris-HCl (pH 7.7), 150 mM NaCl and 5 mM MgCl$_2$) to a final $A_{600}$= 100, frozen in liquid nitrogen and stored at -70°C. Cells were disrupted by two passages through a French press cell at 16 000 p.s.i.. After centrifugation (30 min at 30 000 x g), the supernatant was loaded on a 6-ml Ni-NTA-agarose (Qiagen) column, equilibrated with buffer A. The column was washed with 9 column volumes of buffer A + 20 mM imidazole and 9 column volumes of buffer A + 40 mM imidazole. The protein was eluted with buffer A + 200 mM imidazole. The eluted protein was kept on ice, where it remains active for at least 8 days. Protein concentration was determined according to Lowry et al. (26) using bovine serum albumin as a standard.

**ATPase Assay**

His-tagged MalK was preincubated at 30°C for 5 min in 19 µl of reaction mixture containing 36 mM HEPES-KOH (pH 8.0), 7 mM Tris-HCl (pH 7.7), 27 mM tri-potassium citrate, 15 mM NaCl, 10 mM magnesium acetate, 20 mM imidazole, 1 mM dithiothreitol, 210 µg·ml$^{-1}$ acetylated bovine serum albumin (Sigma) before adding 1 µl [$\gamma^{32}$P] ATP (1 mM, 0.01 Ci/mmol) and further incubating at 30°C. The reaction was stopped by adding 2 µl of 0.25 M EDTA. Aliquots (2 µl) were spotted on PEI cellulose plates (Schleicher and Schüll) and developed in 1 M formic acid and 0.5 M LiCl. The chromatograms were dried and scanned on a PhosphorImager.
Abortive Initiation Assay

MalT was preincubated at 30°C for 15 min in 18 μl of reaction mixture containing 40 mM HEPES-KOH (pH 8.0), 9 mM Tris-HCl (pH 7.7), 27 mM tri-potassium citrate, 17 mM NaCl, 11 mM magnesium acetate, 0.11 mM EDTA, 1.1 mM dithiothreitol, 22 mM imidazole, 230 μg·ml⁻¹ acetylated bovine serum albumin (Sigma), 5 nM malPp DNA fragment, and the indicated concentrations of maltotriose, AMP-PNP and MalK. RNA polymerase solution (2 μl) (0.13 μM in 40 mM HEPES-KOH (pH 8.0), 33 mM tri-potassium citrate, 1 mM dithiothreitol, and 0.1 mg·ml⁻¹ acetylated bovine serum albumin) was added, and the mixture was incubated for 15 min at 30°C. The synthesis of abortive products (ApApC) was initiated by adding 2 μl of a solution containing 5 mM ApA, 0.5 mM [α-³²P]CTP (0.2 Ci·mmol⁻¹) and 500 μg·ml⁻¹ heparin (H-0880; Sigma) and allowed to proceed for 15 min at 30°C (heparin blocks open complex formation by trapping free RNA polymerase). The reaction products were separated from free [α-³²P]CTP by chromatography on Whatman 3MM paper as described (27). The chromatograms were dried and scanned on a PhosphorImager, and the amount of ApApC synthesized was quantified. The malPp DNA template is a 320 bp fragment containing the malPp promoter (from -154 to +130), prepared as described (19).

Maltotriose Binding Assay

The 30 μl reaction mixture contained 40 mM HEPES-KOH (pH 8.0), 7 mM Tris-HCl (pH 7.7), 27 mM tri-potassium citrate, 15 mM NaCl, 10 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, 20 mM imidazole, 2 mM ATP, 0.5 μM [¹⁴C]maltotriose (900 mCi·mmol⁻¹), 0.2 mg·ml⁻¹ acetylated bovine serum albumin, and MalT and MalK as indicated. After 10 or 30 min of incubation at 20°C, the tube was chilled on ice for 5 min and the proteins were precipitated by adding 180 μl of a solution containing 3.1 M (NH₄)₂SO₄, 40 mM HEPES-KOH (pH 8.0), 33 mM tri-potassium citrate, 10 mM
magnesium acetate, 0.1 mM EDTA, 1 mM DTT and 2 mM ATP. After 5 min on ice, the precipitate was collected by a 10-min centrifugation in a microcentrifuge at 4°C, washed with 120 µl of the same solution, dissolved in 500 µl of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and counted in 5 ml of liquid scintillation mixture for 5 min. All values represent the average of assays performed in duplicate and are corrected for the background level (60 cpm) measured in the absence of MalT and MalK. The variations observed between two assays did not exceed 13%.

**Affinity Chromatography with immobilized MalK**

Bacteria overproducing His-tagged MalK+ or MalK-G346S were prepared as for MalK purification. A 10-ml cell suspension (A₆₀₀=100) was thawed, disrupted in a French press cell (16 000 p.s.i.) and centrifuged (60 min at 20 000 x g) to collect the supernatant. Affinity chromatography was performed at 4°C in Micro Biospin® Bio-Rad columns packed with 50 µl of Ni-NTA-agarose (Qiagen). Solutions were passed through the columns by spinning at 5 g for 30 sec in a benchtop centrifuge. The columns were equilibrated with buffer A and loaded with 2 ml of soluble extract. The columns were washed with buffer A (5 x 100 µl), and buffer A + 40 mM imidazole (5 x 100 µl). The MalK-loaded column was then equilibrated with buffer A + 2 mM ATP ± 10 mM maltotriose (2 x 100 µl), and purified MalT (200 µl at 0.5 mg.ml⁻¹ in buffer A + 2 mM ATP (± 10 mM maltotriose) was allowed to flow through the column. Unbound proteins were washed out with 5 x 100 µl of buffer A + 40 mM imidazole + 2 mM ATP (± 10 mM maltotriose). His-tagged MalK was eluted with 4 x 100 µl of buffer A + 500 mM imidazole. 100-µl fractions were collected, starting from the MalT washing step and analyzed by 12% SDS-PAGE (acrylamide:bisacrylamide, 37.5:1).
Affinity chromatography with immobilized DT1

Soluble extracts containing DT1S or DT1S T38R were prepared from BL21(λDE3) ΔmalT220 harboring pOM163 or pOM163 T38R, as described (20). Affinity chromatography was performed at 4 °C in Micro Biospin® Bio-Rad columns packed with 50 µl of Strep-Tactin® Sepharose® resin (IBA), as described above. The columns were equilibrated with buffer B (50 mM Tris-HCl (pH 7.7), 10 % sucrose, 10 mM MgCl₂, and 2 mM AMP-PNP) + 0.5 M KCl, and loaded with 250 µl of DT1S or DT1S-T38R soluble extract supplemented with 2 mM AMP-PNP. The columns were washed with 6 x 100 µl of buffer B + 0.1 M KCl. Freshly purified MalK (1 ml at 0.25 mg/ml, in the washing buffer) was allowed to flow through the DT1-loaded column, and unbound proteins were washed out with 5 x 100 µl of the washing buffer. DT1S or DT1S-T38R was eluted with 4 x 100 µl of buffer B + 0.1 M KCl + 2.5 mM desthiobiotin (Sigma-Aldrich). Ten 100-µl fractions were collected, starting with the MalK washing step and analyzed by 12% SDS-PAGE (acrylamide:bisacrylamide, 37.5:1).
RESULTS

*MalK inhibits transcription activation by MalT in vitro*

We first examined whether purified MalK inhibits MalT in an in vitro transcription system. MalT activity was assayed by monitoring its ability to activate open complex formation at *malPp*, a MalT-dependent promoter, in the presence of RNA polymerase. MalT was incubated with *malPp* DNA and AMP-PNP in the presence or absence of MalK, before adding RNA polymerase and allowing open complex formation. The amount of open complexes formed was then determined by measuring the rate of abortive product synthesis. The assay was performed in the presence of AMP-PNP instead of ATP to avoid MalK and MalT driven ATP hydrolysis, which might interfere with the assay. In addition, given that MalT-dependence on maltotriose is only partial in the presence of AMP-PNP, maltotriose was omitted from the assay. This avoided the possibility that maltotriose might impede repression by MalK. Note also that for a given concentration of MalT, the fraction of the protein that is in the active form, as judged by the promoter response, depends on the concentrations of the positive effectors and on the MalT variant assayed. Therefore, for each combination of effectors and MalT variant used in this work, we determined an individual response curve, i.e. the amount of open complexes formed, as a function of MalT concentration. The concentration of MalT that was used to test the effect of MalK was that eliciting about half of the maximum response under the chosen conditions. The presence of a limiting concentration of MalT in the assay ensures that MalT inhibition by MalK can be readily detected.

As shown in Fig. 1, MalK strongly depressed *malPp* activation by MalT in the absence of maltotriose; the amount of open complexes formed was reduced by 80% in the presence of 8 µM MalK. Furthermore, the inhibitory effect of MalK disappeared when the assay was performed in the presence of 0.1 mM maltotriose (Fig. 1). The fact that
maltotriose antagonizes MalT inhibition by MalK, as expected based on in vivo data, provides evidence that the inhibitory effect observed in vitro is physiologically relevant.

**malT and malK mutations relieving MalT inhibition by MalK in vivo also suppress the inhibitory effect of MalK in vitro**

To confirm that the inhibition caused by MalK in vitro is functionally significant, we examined whether two different malT mutations that are known to confer resistance to MalK in vivo (malT'26 and malT-T38R) have the same effect in vitro. The malT'26 mutation, which generates the R242P substitution in the DT1-DT2 linker, increases the affinity of MalT for maltotriose by favoring the transition from the inactive state to the active state (9,21). It also suppresses MalT-sensitivity to MalK in vivo (22). The T38R substitution, which is located in DT1, diminishes MalT-sensitivity to MalK in vivo (22). As shown in Fig. 2A, neither of these MalT variants was affected by MalK when assayed in the presence of AMP-PNP alone, i.e. under conditions in which wild-type MalT is inhibited by MalK.

We also tested whether the inhibitory effect associated with purified MalK was affected by the malK-G346S mutation. The G346S substitution, which is thought to alter the MalT-binding site, specifically decreases the ability of MalK to down-regulate MalT in vivo without damaging its ability to catalyse maltose transport both when overproduced or when constitutively expressed from a chromosomal locus ((10); E.R., unpublished results). As expected, MalT is not inhibited by MalK-G346S in the presence of AMP-PNP alone (Fig. 2B). To rule out the possibility that the lack of effect of MalK-G346S was caused by protein misfolding, we verified that the purified MalK variant hydrolyses ATP in vitro at exactly the same rate as the wild-type protein (0.32 and 0.33 molecule of ATP are hydrolysed at 30°C per min per protomer of wild-type and mutant protein, respectively). The uncoupled ATPase activity of MalK is a good indicator of correct protein folding, given that ATP hydrolysis by MalK relies on protein dimerization.
and that dimerization involves both the nucleotide binding domain and the regulatory domain, as revealed by the X-ray structure of MalK (28, 29).

Repression of MalT activity by MalK involves a direct interaction between MalK and MalT

Having established an in vitro repression system mimicking in vivo events, we characterised the repression process and tested the simple hypothesis that MalT inhibition by MalK involves formation of a MalK/MalT complex. To do this, we examined whether His-tagged MalK protein immobilized on a Ni-NTA-agarose column would bind the activator. Purified MalT was run over a MalK-loaded column and, after extensive washing, MalK was eluted with 500 mM imidazole. The affinity chromatography was performed in the presence of ATP (ATP hydrolysis was not expected to interfere with the assay because it was present at a high concentration). SDS-PAGE analysis of the collected fractions and Coomassie blue-staining of the gel revealed that a significant fraction of the loaded MalT protein was retained on the column and coeluted with MalK (Fig. 3A). In contrast, MalT was not retained on the MalK-loaded column when the affinity chromatography was performed in the presence of ATP and 10 mM maltotriose (Fig. 3B). Likewise, the T38R MalT variant, which is insensitive to repression by MalK, did not bind MalK, nor does wild-type MalT bind MalK-G346S, the MalK variant defective in MalT regulation (Fig. 3C, D). These data provide compelling evidence that MalT physically interacts with MalK, that the interaction is functionally relevant, and that maltotriose prevents formation of the MalT/MalK complex.

MalK antagonizes inducer binding by MalT

We then tested whether, reciprocally, MalK would inhibit maltotriose binding by MalT. MalT was incubated in the presence of ATP, labelled maltotriose and MalK, and the amount of bound maltotriose was measured by precipitating the proteins with ammonium
sulfate. As shown in Fig. 4, wild-type MalK markedly reduced maltotriose binding by wild-type MalT whereas it had no significant effect on maltotriose binding by MalT26 or MalT-T38R. The same data were obtained for 10-min and 30-min incubation times, which excludes the possibility that the differences between the responses of wild-type MalT and the mutated proteins were due to kinetic effects. We verified that the concentrations of wild-type MalT, MalT26 and MalT-T38R in the assays were limiting, i.e. that the absence of inhibitory effect was meaningful (data not shown). Furthermore, as predicted, G346S MalK does not prevent maltotriose binding by wild-type MalT (Fig. 4). Based on these results, we conclude that MalK inhibits maltotriose binding by MalT.

MalK interacts with DT1
The similarity between the action modes of the three negative effectors of MalT suggests that, like MalY and Aes, MalK might interact with DT1. To test this hypothesis, we examined whether immobilized DT1 binds the MalK protein. Purified MalK was chromatographed in the presence of AMP-PNP on a Strep-Tactin® Sepharose® column preloaded with the C-terminally Strep-tagged DT1 domain (DT1S), as previously described (20). As shown in Fig. 5A, a significant fraction of the wild-type MalK protein was retained on the column and was recovered with DT1S upon elution with desthiobiotin. Furthermore, no retention was observed when wild-type MalK was replaced by the G346S variant (Fig. 5B). We therefore infer that MalK interacts specifically with DT1.

Since the T38R substitution is located in DT1, we considered the possibility that this amino-acid substitution renders MalT insensitive to MalK by altering the DT1 determinant recognized by MalK. Affinity chromatography experiments performed with immobilized DT1S-T38R unexpectedly revealed that the mutated domain still binds wild-type MalK as well as MalK-G346S (Fig. 5C, D). These results demonstrate that the T38R
substitution does not prevent MalK binding to isolated DT1 although it modifies the DT1 determinant recognized by MalK so that it does not respond any more to the malK-G346S mutation. Yet full length MalT-T38R does not bind MalK (Fig. 3C); the effect of the T38R substitution on the interaction between MalK and full-length MalT must thus be indirect and probably involves a conformational effect.
DISCUSSION

The work presented here clearly establishes that the regulatory action of MalK on MalT is direct and does not require any additional factor. It also demonstrates that MalK inhibits MalT by interacting with the activator and by antagonizing inducer binding. These conclusions are based on the following observations: (i) purified MalK is able to prevent open complex formation at the malPp promoter in the presence of RNA polymerase in a purified transcription system; (ii) as shown by affinity chromatography, MalK specifically interacts with MalT; and (iii) MalK inhibits maltotriose binding. Most importantly, the inhibitory effect observed in vitro is relieved by two malT mutations (malT’26 and malT-T38R) and by one malK mutation (G346S) that are known to suppress MalT repression by MalK in vivo. This provides compelling evidence that our in vitro repression system is of physiological relevance. Finally, we have demonstrated that, when isolated, DT1 specifically interacts with MalK, which indicates that part, if not all, of the MalK binding site is located on DT1, the N-terminal domain of MalT. Yet, the MalT/DT1 interaction is weak, which suggests that other MalT domains might contribute, directly or indirectly, to MalK binding.

It might seem surprising that the MalT/MalK interaction revealed here by the affinity chromatography experiments performed with immobilized MalK responds to maltotriose or to the malT’26 mutation whereas this was not the case in similar experiments that were performed with immobilized MalT by Panagiotidis et al. (11). However, in their experiments the immobilized MalT protein might be unfolded due to the absence of ATP during the extraction process (30). As a result, the ability of MalT to interact with MalK was possibly unregulated and the secondary structural motifs involved in MalK recognition permanently exposed. As discussed below, maltotriose and malT’ mutations such as malT’26 are indeed not expected to interfere with MalK binding through direct competition or by altering the MalK binding site. Instead, they probably
antagonize MalK binding indirectly by stabilizing the active form of MalT, i.e. the form in which the MalK binding site is either masked or has a reduced affinity for MalK.

Our finding that, like MalY, MalK specifically interacts with DT1 in vitro may seem at odds with the previous observation that DT1 does not titrate MalK in vivo while its does titrate MalY (22). The discrepancy between the two sets of data might stem from the fact that the experiments were not performed with the same form of DT1. The DT1 polypeptide used in vitro was Strep-tagged at its C-terminus whereas the DT1 form used in vivo was fused to the TrxA polypeptide at its N-terminus. The TrxA extension might have interfered specifically with MalK binding.

The malT-T38R mutation turns out to confer complex properties to the MalT protein. The T38R substitution is located in DT1 just upstream from the Walker A motif, which is thought to form a P-loop interacting with the β and γ phosphates of bound ATP (31). Intriguingly, the amino-acid change impairs MalK binding in the full-length MalT context but not in the DT1 context. This obviously indicates that the substitution does not alter the DT1 surface element recognized by MalK, but indirectly interferes with MalK binding via a conformational change involving other MalT domains. Such a long range effect is consistent with the observation that the T38R substitution also alters the interplay between nucleotide binding and inducer binding. The substitution indeed alleviates the requirement for maltotriose, i.e. renders the protein constitutively active, in the presence of ADP (N. J., unpublished). Nevertheless, the T38R MalT substitution also modifies, directly or indirectly, the DT1 determinant recognized by MalK since it makes the DT1/MalK interaction insensitive to mutation malK-G346S in the DT1 context.

An interesting finding of this work is that MalK inhibits MalT via the same mechanism as MalY and Aes (19, 20). Indeed, in all three cases, the negative effector inhibits maltotriose binding and vice versa. In addition, as revealed by the MalT activity assays, a high concentration of maltotriose fully relieves MalT inhibition by MalK (Fig. 1A), MalY or Aes, thereby suggesting a phenomenon of competitive inhibition.
Finally, DT1 is directly involved in the binding of the negative effector in all three cases. Taking all available data into account, the following picture emerges (Fig. 6). MalT is in equilibrium between an inactive and an active form, with the negative effector stabilizing the inactive form and maltotriose stabilizing the active form. The competition between maltotriose and the negative effector would be indirect and occur via an interconversion between an active and an inactive form. In the inactive form, the binding sites for the negative effectors, which are made up, at least in part, of determinants provided by DT1, would be available or correctly configured, while the maltoehoriase binding site would not be accessible. Conversely the active form would offer a high-affinity binding site for maltotriose because of a proper relationship between DT2 and DT3, while the binding sites for the negative effectors would not be exposed or properly configured (21). The interconversion between the inactive and active states involves DT1, -2, and -3, based on the observation that the transition toward the active state causes a change in the sensitivity of both the DT1-DT2 and the DT2-DT3 linkers to protease attack (21). Given that the three inhibitory proteins are unrelated at the sequence level and that MalK and MalY do not share any obvious structural similarity (29,32), the molecular details of the interaction between the inactive form of MalT and each of the negative effectors is likely to be different. Consistent with this idea, the T38R substitution interferes with the DT1/Aes interaction (20) in the DT1 context while it has no effect on the DT1/MalK interaction.

The model proposed is consistent with the observations that mutation malT26 renders MalT insensitive to repression by MalK, MalY(19) or Aes (20). Mutation malT26, which corresponds to the R242P substitution in the DT1-DT2 linker, is indeed known to shift the equilibrium toward the active form of MalT. The mutated protein is partially active in the absence of maltotriose and displays a higher affinity for the inducer compared to the wild-type protein (9). Moreover, in the absence of maltotriose, a fraction of the MalT26 variant exists spontaneously under a conformation characteristic of the
wild-type maltotriose-bound protein, as revealed by mild proteolysis (21). Hence, although we cannot exclude the possibility that the DT1-DT2 linker might be part of the MalK, MalY and Aes binding sites, we favor the idea that the malT26 mutation makes MalT insensitive to the repressor proteins, simply by stabilizing the active form of MalT, i.e. via a long range effect.

Finally, it is worth emphasizing that the multiplicity of the signals sensed by MalT makes this activator unique among prokaryotic transcription factors. In this respect, it will be interesting to understand how all of these signals are integrated at the level of the protein and, in particular, what are the conformational changes involved in the transition between the inactive and the active forms and what role the ATPase activity of MalT plays in the control of this interconversion.

Two important questions remain to be answered. First, the model substrate used here is the soluble form of MalK but, in vivo, MalK associates with MalF and MalG, the two integral membrane components, to form the maltodextrin transporter. Thus, which form of MalK down-regulates MalT in vivo: the MalFGK₂ transporter or a soluble MalK form that would cycle between the membrane and the cytoplasm? Second, how does maltodextrin transport trigger derepression of MalT? Panagiotidis et al. (11) observed that a MalK variant that binds but does not hydrolyse ATP has a super-repressor phenotype whereas MBP-independent transporter variants with high ATPase activity, even in the absence of transportable substrate, show a reduced ability to repress MalT. Based on these observations, they inferred that the regulatory activity of MalK would actually be coupled to its ATPase activity and that the repressive form of MalK would be the ATP-bound form, which is expected to predominate when the transport system is resting. Such a model is not supported by the structural data of Chen et al. (29). Instead, the crystal structures of free and ATP-bound MalK dimers suggest that ATP hydrolysis is not accompanied by conformational changes of the C-terminal, regulatory domain, where the MalT binding-site is located (10). The structural consequences of ATP hydrolysis
might be different however in the context of the assembled transporter, and conformational changes of the MalK regulatory region might accompany the ATP hydrolysis cycle when coupled to transport and thus control the ability of MalK to interact with MalT. Furthermore, it seems unlikely that the repressing function of MalK is constitutive and that the induction of the maltose regulon results solely from reversal of the inhibition by MalK due to an increase in the intracellular concentration of maltotriose upon maltose entrance, given that the concentration of MalK, whose structural gene is part of the maltose regulon, increases concomitantly.
ACKNOWLEDGEMENTS

We are grateful to Olivier Danot for stimulating discussions and his comments on the manuscript, and for providing plasmids pOM163 and pOM163-T38R. We also thank Tony Pusgley for his interest in this work and critical reading of the manuscript. We acknowledge support for part of this work by the “Fonds der chemischen Industrie” and the “Deutsche Forschungsgemeinschaft”. N. J. was the recipient of a followship from the Ministère Délégué à la Recherche et aux Nouvelles Technologies.
REFERENCES


FOOTNOTES

1 AMP-PNP, which can replace ATP as an effector of MalT (33), is actually more effective than ATP in driving MalT self-association in the presence of maltotriose due to the absence of ATP hydrolysis, which generates ADP-bound forms that are less prone to multimerisation (16).

2 MalT is 50% as active in the presence of AMP-PNP alone as in the presence of AMP-PNP and a saturating concentration of maltotriose (N. J., unpublished results).
FIGURE LEGENDS

**Fig. 1. Effect of MalK on MalT activity in an in vitro system.** Abortive initiation assays were performed in the presence of 0.5 mM AMP-PNP and the indicated concentrations of maltotriose, wild-type MalT and wild-type MalK. Closed squares, no maltotriose and 240 nM MalT; open diamonds, 0.1 mM maltotriose and 120 nM MalT. All of the assays were performed in duplicate. The inset shows the responses to increasing concentrations of MalT obtained in the presence of 0.5 mM AMP-PNP ± 0.1 mM maltotriose. The sigmoidicity of the curve reflects cooperative binding of MalT to the three MalT operator sites involved in *malPp* activation (15).

**Fig. 2. Effect of *malT* and *malK* mutations on MalT repression by MalK.** Abortive initiation assays were performed in the presence of 0.5 mM AMP-PNP and the indicated concentrations of MalT and MalK variants. A, closed squares, 240 nM MalT26; open diamonds, 300 nM MalT-T38R. B, closed squares, 240 nM wild-type MalT ± wild-type MalK; open diamonds, 240 nM wild-type MalT ± MalK-G346S.

**Fig. 3. Affinity chromatography on a MalK-loaded column.** Purified MalT was chromatographed on Ni-NTA-agarose microcolumns preloaded with His-tagged MalK, in the presence of 2 mM ATP, as described in Experimental Procedures. FT, W and E stand for the flow-through (200 µl), wash fractions (100 µl each) and imidazole eluted fractions (100 µl each), respectively. A, Chromatography of wild-type MalT on a column preloaded with His-tagged MalK. B, Chromatography of wild-type MalT on a column preloaded with His-tagged MalK with 10 mM maltotriose in the chromatography buffer besides ATP. C, Chromatography of MalT-T38R on a column preloaded with His-tagged MalK. D, Chromatography of wild-type MalT on a column preloaded with His-tagged MalK-G346S.
Fig. 4. Effect of MalK on maltotriose binding by MalT. Maltotriose binding assays were performed at 0.5 μM maltotriose with wild-type MalT (2 μM), MalT26 (1 μM) or MalT-T38R (1.5 μM) in the presence of 19 μM wild-type MalK or MalK-G346S, as indicated. Incubation at 20°C was for 10 min (black bars), or 30 min (open bars).

Fig. 5. Affinity chromatography on a DT1-loaded column. Purified MalK (1 ml at 0.25 mg/ml) was chromatographed on Strep-tactin® Sepharose® microcolumns preloaded with wild-type DT1S or the DT1S-T38R variant, in the presence of 2 mM AMP-PNP as described in Experimental Procedures. FT, W and E stand for the flowthrough (200 μl), wash fractions (100 μl each) and desthiobiotin eluted fractions (100 μl each), respectively. A, Wild-type DT1S and wild-type MalK. B, Wild-type DT1S and MalK-G346S. C, DT1S-T38R and wild-type MalK. D, DT1S-T38R and MalK-G346S.

Fig. 6. A model for the interplay between the inducer and the negative effectors of the MalT. The monomeric, inactive MalT form (Tᵈ) is in equilibrium with the monomeric, active form (Tᵦ), which is prone to multimerization. The negative effectors, MalK, MalY or Aes, stabilize the inactive form whereas maltotriose stabilizes the active form. The multimeric forms are the transcriptionally competent forms responsible for recognition of the target promoters. The equilibrium between the various MalT species also depends on whether MalT is liganded to ATP or ADP. ATP-bound MalT is more prone to multimerization in the presence of maltotriose than the ADP-bound form (16). In contrast, as shown for MalY and Aes, inhibition of ADP-bound MalT by MalY or Aes is insensitive to the presence of an excess of maltotriose (19, 20).
FIG. 1.
FIG. 2.
**Fig. 3.**

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**A** wt MalK / wt MalT

**B** wt MalK / wt MalT + triose

**C** wt MalK / MalT T38R

**D** MalK-G346S / wt MalT
Fig. 4.
FIG. 5.
FIG. 6.
Additions and Corrections


MalK, the ATP-binding cassette component of the *Escherichia coli* maltodextrin transporter, inhibits the transcriptional activator MalT by antagonizing inducer binding.

*Nicolas Joly, Alex Böhm, Winfried Boos, and Evelyne Richet*

Page 33124, lines 7 and 8 under “Experimental Procedures”: The name and sequence of the upstream primer used to amplify the *malK* gene, rather than KU001 (5'-GCCGCCATGGGGACCCACGATCAGGTCGAC-3'), should be: KU006 (5'-CCGCCCATGGGGATGGCGAGCGTACAGCTGC-3').


Binding specificity of sea anemone toxins to Na*, 1.1–1.6* sodium channels. Unexpected contributions from differences in the IV/S3-S4 outer loop.

*Joacir Stolarz Oliveira, Elisa Redaelli, André J. Zaharenko, Rita Restano Cassulini, Katsuhiko Konno, Daniel C. Pimenta, José C. Freitas, Jeffrey J. Clare, and Enzo Wanke*

Page 33323, line 10 of the summary: L36A should be changed to K36A.

Page 33331, next to the last line in the left-hand column: The first Asp-Ile should be changed to Asp-Leu, to read “The Asp-Leu instead of the Asp-Ile motif . . .”

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
R-cadherin influences cell motility via Rho family GTPases.

Emhonta Johnson, Christopher S. Theisen, Keith R. Johnson, and Margaret J. Wheelock

Page 31046: The wrong Fig. 5 was printed. The correct figure is shown below:
MalK, the ABC component of the Escherichia coli maltodextrin transporter, inhibits the transcriptional activator MalT by antagonizing inducer binding
Nicolas Joly, Alex Böhm, Winfried Boos and Evelyne Richet

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