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Characterization of a Novel Prokaryotic GDP Dissociation Inhibitor Domain from the G Protein Coupled Membrane Protein FeoB

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The FeoB family of membrane embedded G proteins are involved with high affinity Fe(II) uptake in prokaryotes. Here, we report that FeoB harbors a novel GDP dissociation inhibitor-like domain that specifically stabilizes GDP-binding through an interaction with the switch I region of the G protein. We show that the stabilization of GDP binding is conserved between species despite a high degree of sequence variability in their guanine nucleotide dissociation inhibitor (GDI)-like domains, and demonstrate that the presence of the membrane embedded domain increases GDP-binding affinity roughly 150-fold over the level accomplished by action of the GDI-like domain alone. To our knowledge, this is the first example for a prokaryotic GDI, targeting a bacterial G protein-coupled membrane process. Our findings suggest that Fe(II) uptake in bacteria involves a G protein regulatory pathway reminiscent of signaling mechanisms found in higher-order organisms.

Keywords: bacterial proteins; GTP-binding protein; guanine nucleotide dissociation inhibitor; switch region; Fe(II)-uptake

Introduction

Small GTP-binding proteins are well known for their ubiquitous contribution to signal transduction in eukaryotic cells.[1,2] In contrast, the mechanistic contribution of prokaryotic G proteins to bacterial physiology remains largely unresolved because in many cases understanding of these G proteins has not advanced past association with global processes, such as ribosome biogenesis, tRNA modification, progression through the cell cycle or DNA replication.[3–6]

The advent of several near atomic resolution structures of prokaryotic G proteins[7–10] show that these G proteins are structural twins of their eukaryotic relatives. While very helpful in establishing commonalities between prokaryotic and eukaryotic G proteins, the structures have not been able to explain why the nucleotide binding affinities of the majority of prokaryotic GTPases are several orders lower than those observed in their eukaryotic counterparts, nor did the structures provide insights into the molecular mechanism by which most prokaryotic G proteins act within bacterial cells. Addressing these issues, work on the signal recognition particle protein FtsY suggested that prokaryotic G proteins are regulatory and that fast nucleotide release is promoted by a domain N-terminal to the GTPase that may act like an intrinsic guanine nucleotide exchange factor (GEF).[11,12] While very attractive, this hypothesis has not been verified experimentally, nor have distinct GEFs been identified. Similarly, no GTPase activating proteins (GAPs), which stimulate GTP hydrolysis, or guanine

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Abbreviations used: GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; GDI, guanine nucleotide dissociation inhibitor; mant, *N*-methylanthraniloyl; GMPPNP, guanosine-5′[(β,γ)-imido]triphosphate; MBP, maltose binding protein; LB, Luria broth.

nucleotide dissociation inhibitor (GDI)-like interaction partners, which inhibit nucleotide dissociation, have been found that would establish a prokaryotic version of the regulatory G protein cycle, with exception of EF-Tu and EF-Ts that are involved in translation. Apart from the latter two, toxins that are secreted by pathogenic bacteria to diminish host cell resistance to infection through interference with eukaryotic G proteins are the only other documented examples of prokaryotic G protein effectors.[13,14]

Prokaryotic uptake of ferrous iron recently emerged as a G protein coupled process involving the membrane protein FeoB, whose soluble, intracellular N-terminal region combines a GTP-binding/GTPase domain and a spacer domain of unknown function.[15] Compared to TrmE, Era and EngA, FeoB provides a narrowly defined physiological readout and thus can be exploited to address structure–function relationships in the TrmE-Era-EngA-YihA-Septin-like superfamily of G proteins, which includes several prokaryotic members of the translation factor class of G proteins.[16]

Here we show that just like eukaryotic G proteins, integrity of the switch regions is important for function of the GTP-binding domain of FeoB. Moreover, we show that GDP binding is stabilized through an interaction of the switch regions with a highly variable linker domain (IIFeoB) that connects the GTP-binding domain (IFeoB) to the membrane-embedded part of FeoB (IIIFeoB). We furthermore provide evidence that the presence of the membrane-embedded domain further increases GDP affinity, possibly by stabilizing IIFeoB binding to the switch regions of the GTP-binding domain. Taken together, these findings suggest that the ancient process of ferrous iron uptake involves a crude blueprint for a G protein cycle that over time has evolved into a central regulatory element of cell function in higher organisms.

Results

NFeoB has a higher affinity for GDP than GTP

In our previous study we used fluorescently labeled *N*-methylanthraniloyl (mant)- guanosine-5′[(β,γ)-imido]triphosphate (GMPPNP), a non-hydrolyzable GTP analog, to estimate GTP binding affinity.[15] When it became apparent that NFeoB (Figure 1(a); residues 1–274 in *Escherichia coli*) may serve a regulatory function, we decided to re-visit our original nucleotide-binding experiments, and to explicitly measure mant-GTP affinity instead of using mant-GMPPNP affinity as a substitute. Given that the k_{cat} value of GTP hydrolysis was 0.0015 s^{-1} at 37 °C (Table 1), it seemed unlikely that GTP hydrolysis would constitute a problem during stopped-flow experiments whose duration was limited to a few seconds. To our surprise, we found that in the case of NFeoB, mant-GMPPNP was not a good indicator for GTP affinity as its dissociation constant was about an order of magnitude lower than the K_d for mant-GTP, which was determined to be 12 μM. Interestingly, the K_d value for mant-GTP also was higher than the mant-GDP K_d previously reported (and confirmed here to be 4.5 μM at 10 °C (Table 1)), thus reversing the relative binding affinity reported to mirror that of other small regulatory G proteins (= $K_{d(GDP)} < K_{d(GTP)}$). Nevertheless, NFeoB's nucleotide binding properties still resembled the Era family of bacterial GTPases, in that it featured a fast and spontaneous release of GDP coupled to slow GTP hydrolysis.[17] Notably, even the amended binding affinities would be consistent with the previous suggestion that under physiological conditions (~0.1 mM for GDP, 1 mM for GTP[18]), NFeoB, but not full-length FeoB as will be shown later (Table 2), would be mostly in its GTP-bound form. To further test this hypothesis, which relied on the assumption that the binding affinity roughly doubles every increase of 10 °C,[15] and to ensure that stopped-flow studies of mant-modified guanine nucleotides are representative of the true binding affinities, we performed isothermal calorimetry to measure GDP binding directly (data not shown). The values determined for GDP were consistent with the interpolated values and suggested that the presence of the mant group did not have a large impact on the nucleotide affinity (data not shown).

Switch I and II regions are important for function

The switch regions of GTP-binding proteins are important in processing guanine nucleotides by interacting with downstream effectors and adopting unique conformations dependent upon what nucleotide species is bound.[19] To assess the significance of the switch regions, we tested whether targeted point mutations interfered with *feoB* function *in vivo* and whether the phenotypic behavior of these mutants could be understood through changes in the nucleotide binding and enzymatic properties of NFeoB, the soluble N-terminal domain of FeoB (Figure 1(a)). Six mutants in the switch regions, two in switch I (N32A, T37A) and four in switch II (T60Q, Y61E, Y61A, D73A), were analyzed (Figure 1(b)). The mutations targeted residues/regions known to play important roles at different stages within the nucleotide cycle (GTP/GDP-binding, catalysis, and effector interaction) of regulatory G proteins. In switch I, Asn32 is one of the most highly conserved residues and analogous mutations in p21ras are functionally important.[20] The choice of Thr37 was motivated by its contribution to the coordination of a Mg^{2+} that through interactions with the nucleotide as well as the switch regions brings together multiple parts of the nucleotide binding site.[21] In switch II, Thr60 was targeted because the corresponding glutamine residue in p21ras is thought to contribute to catalysis, but not conserved in FeoB. As for the highly conserved Tyr61 and Asp73, the former is adjacent to the G3 motif that is involved in coordination of the phosphates, while Asp73 maps to the part of switch II that undergoes nucleotide-dependent structural rearrangements in eukaryotic