Reciprocal Stimulation of GTP Hydrolysis by Two Directly Interacting GTPases

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The *Escherichia coli* guanosine triphosphate (GTP)–binding proteins Ffh and FtsY have been proposed to catalyze the cotranslational targeting of proteins to the bacterial plasma membrane. A mutation was introduced into the GTP-binding domain of FtsY that altered its nucleotide specificity from GTP to xanthosine triphosphate (XTP). The mutant FtsY protein stimulated GTP hydrolysis by a ribonucleoprotein consisting of Ffh and 4.5*S* RNA in a reaction that required XTP, and it hydrolyzed XTP in a reaction that required both the Ffh-4.5*S* ribonucleoprotein and GTP. Thus, nucleotide triphosphate hydrolysis by Ffh and FtsY is likely to occur in reciprocally coupled reactions in which the two interacting guanosine triphosphatases act as regulatory proteins for each other.

Guanosine triphosphatases (GTPases) control and regulate many biological processes, including translation, signal transduction, cytoskeletal organization, vesicle transport, nuclear import, and protein translocation across membranes (*1*). In many cases, multiple GTPases are linked together within sophisticated pathways or cascades (*2*). Through GTP binding and hydrolysis, GTPases can exist in two discrete conformations, a GTP-bound state and a guanosine diphosphate (GDP)–bound state, that together define the basic GTPase "switch" (*3*). Interconversion between these two states allows the GTPase to interact in temporal succession with other macromolecules to regulate and direct a given biological process. The rate of conversion between the GTP-bound and GDP-bound states of most GTPases is modulated by external effectors, including guanine nucleotide release factors (GNRFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (*4*). Here, we report a situation in which two interacting GTPases modulate each other's GTPase activities.

Ffh and FtsY are related proteins from *E. coli* that are homologous to essential components of the eukaryotic signal recognition particle (SRP) and SRP receptor, respectively. Both proteins contain GTP-binding domains that define a distinct subgroup in the superfamily of GTPases (*5*). In addition, FtsY contains an NH_2-terminal domain of unknown function, and Ffh contains a COOH-terminal domain through which it is tightly bound to 4.5*S* RNA (*6*). In vitro, the Ffh-4.5*S* ribonucleoprotein (RNP) has a weak GTPase activity on its own, whereas no measurable GTPase activity has been detected for FtsY alone (*7*). When Ffh-4.5*S* RNP and FtsY are combined, however, a greatly stimulated GTPase activity results (*7*). To explore this reaction in more detail, we followed the rate of GTP hydrolysis as a function of GTP concentration (Fig. 1A). The reaction catalyzed by the Ffh-4.5*S* RNP alone was linear on an Eadie-Scatchard plot, which would indicate simple Michaelis-Menten behavior (Fig. 1B). In contrast, the stimulated reaction that contained both Ffh-4.5*S* RNP and FtsY gave a hyperbolic curve, with the maximum occurring at ~0.5 μM GTP (Fig. 1C). This result would be consistent with a model for a cooperative dimer, in which the binding of substrate to one component would increase the binding of substrate to a second component (*8*). Because both Ffh and FtsY are GTP-binding proteins, however, this analysis could not distinguish whether FtsY only modulated the GTPase activity of Ffh-4.5*S* RNP or whether FtsY also bound to and hydrolyzed GTP.

To investigate the role of nucleotide binding to FtsY, we took advantage of the observation that a single Asp → Asn substitution in the translation elongation factor EF-Tu alters its nucleotide specificity from GTP to xanthosine triphosphate (XTP) (*9*, *10*). This Asp residue, located in box IV of the GTP-binding consensus motif (NXKD) (*11*), is conserved throughout the superfamily of GTPases, including FtsY (*1*). We introduced the Asp → Asn mutation into the gene for FtsY, and the resulting mutant protein, termed FtsY(D441N), was expressed as a fusion protein with glutathione-S-transferase (GST) and purified for use in GTPase assays (*12*).

We first tested the ability of FtsY(D441N) to stimulate the GTPase of Ffh-4.5*S* RNP in the presence and absence of XTP (Fig. 2). The GTP concentration (0.5 μM) used in this experiment corresponded to the maximum on the Eadie-Scatchard plot (Fig. 1C). In the absence of XTP, no stimulation of GTP hydrolysis by FtsY(D441N) was observed beyond that catalyzed by Ffh-4.5*S* RNP alone, but when XTP was added, the rate of GTP hydrolysis increased; at a concentration of 10 μM XTP, the rate became similar to that afforded by wild-type FtsY (Fig. 2A). Xanthosine diphosphate (XDP) was unable to substitute for XTP in stimulating the Ffh-4.5*S* RNP GTPase (Fig. 2B) and inhibited the XTP-stimulated GTPase reaction (Fig. 2C). Thus FtsY(D441N) needed to be in a nucleotide triphosphate–bound state to stimulate the Ffh-4.5*S* RNP GTPase. To control for the specificity of XTP binding to FtsY(D441N), we analyzed the ability of other purine nucleotide triphosphates to stimulate the Ffh-4.5*S* RNP GTPase (Fig 2B). In this reaction, adenosine triphosphate (ATP) showed no effect. In contrast, inosine triphosphate (ITP) gave a modest stimulation in GTPase activity, consistent with the observation made for the analogous mutation in EF-Tu (*9*).

We next assayed the behavior of FtsY(D441N) in the Ffh-4.5*S* RNP–dependent GTPase reaction at 10 μM XTP and at various concentrations of GTP (Fig. 3). In the presence of XTP, FtsY(D441N) caused greater stimulation than did wild-type FtsY at concentrations of GTP below 0.5 μM (Fig. 3A). This finding was consistent with the need for FtsY to have bound nucleotide triphosphate to stimulate the Ffh-4.5*S* RNP

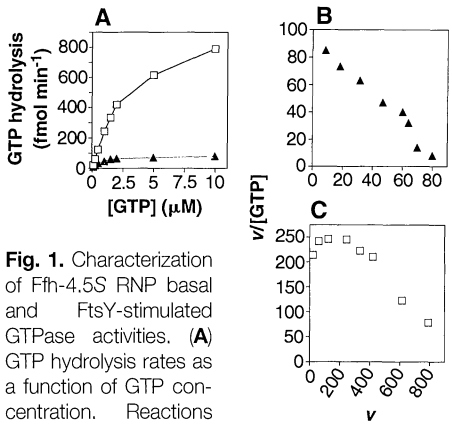

Fig. 1. Characterization of Ffh-4.5*S* RNP basal and FtsY-stimulated GTPase activities. (**A**) GTP hydrolysis rates as a function of GTP concentration. Reactions contained 5 nM Ffh-4.5*S* RNP (▲) or 5 nM Ffh-4.5*S* RNP + 150 nM FtsY-GST (□) in 20 μl of buffer containing 25 mM triethanolamine acetate (pH 7.5), 2.5 mM magnesium acetate, 25 mM potassium acetate, 1 mM dithiothreitol, 0.1% Nikkol, and 5% glycerol. [γ-^{32}P]GTP (ICN Biochemicals, Irvine, CA) was added, along with cold GTP, to the appropriate final concentration. After incubation for 20 min at 25°C, the phosphate liberated during the reaction was determined by Cerenkov counting, as described (*7*). Data points correspond to 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 5.0, and 10.0 μM GTP. The Michaelis constant of the Ffh-4.5*S* RNP GTPase was ~0.7 μM. Ffh-4.5*S* RNP and FtsY-GST were purified as described (*7*, *17*). (**B** and **C**) Data from (A) are presented in the form of Eadie-Scatchard plots (*v*, GTP hydrolysis in femtomoles per minute) for (B) Ffh-4.5*S* RNP and (C) FtsY + Ffh-4.5*S* RNP. The maximum in (C) occurs at ~0.5 μM GTP.

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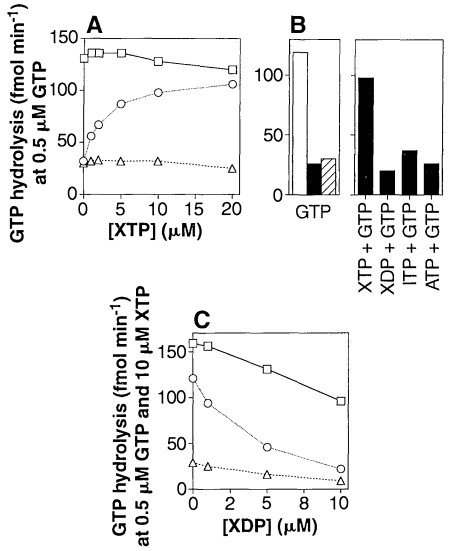

Fig. 2. Characterization of FtsY(D441N) in GTPase assays. (**A**) GTP hydrolysis rates as a function of XTP concentration. □, FtsY + Ffh-4.5*S* RNP; ○, FtsY(D441N) + Ffh-4.5*S* RNP; △, Ffh-4.5*S* RNP. (**B**) Specificity of FtsY(D441N) for XTP in stimulating Ffh-4.5*S* RNP GTPase. GTP was present at 0.5 μM; all other nucleotides were present at 10 μM. Open bar, FtsY + Ffh-4.5*S* RNP; solid bars, FtsY(D441N) + Ffh-4.5*S* RNP; striped bar, Ffh-4.5*S* RNP. (**C**) Inhibition by XDP of the XTP-stimulated hydrolysis of GTP by FtsY(D441N) + Ffh-4.5*S* RNP. Reactions were performed as described in Fig. 1. Symbols are the same as in (A).

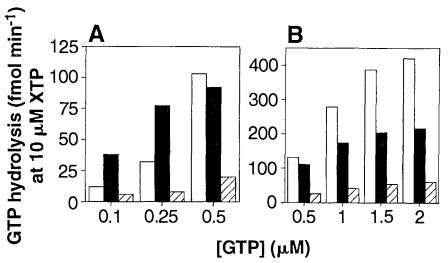

Fig. 3. Behavior of FtsY(D441N) at different GTP concentrations. (**A**) 0.1 to 0.5 μM GTP; (**B**) 0.5 to 2.0 μM GTP. The concentration of XTP was 10 μM in all cases. Reactions were performed as described in Fig. 1. Open bars, FtsY + Ffh-4.5*S* RNP; solid bars, FtsY(D441N) + Ffh-4.5*S* RNP; striped bars, Ffh-4.5*S* RNP.

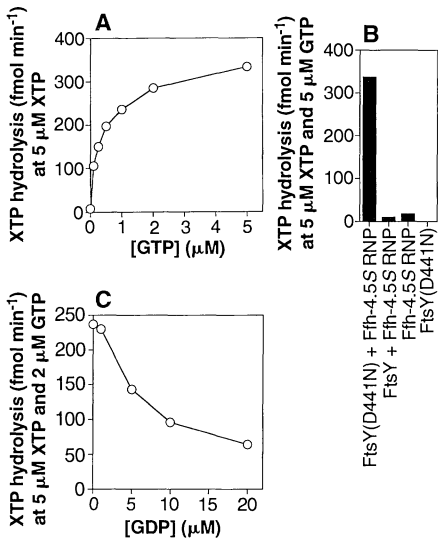

Fig. 4. FtsY(D441N) hydrolyzes [γ-^{32}P]XTP in a reaction that depends on both Ffh-4.5*S* RNP and GTP. (**A**) XTP hydrolysis rates as a function of GTP concentration in the reaction that contained FtsY(D441N) + Ffh-4.5*S* RNP. (**B**) Control reactions demonstrating that only FtsY(D441N) + Ffh-4.5*S* RNP hydrolyzes XTP. (**C**) Inhibition by GDP of the GTP-stimulated hydrolysis of XTP by FtsY(D441N) + Ffh-4.5*S* RNP. Reactions were performed as described in Fig. 1, except that enzymatically prepared [γ-^{32}P]XTP was used (*18*).

GTPase and would also explain the shape of the Eadie-Scatchard plot for the reaction that contained FtsY (Fig 1C): At low concentrations of GTP and saturating concentrations of XTP, FtsY (D441N) was "primed" better than wild-type FtsY to stimulate the Ffh-4.5*S* RNP GTPase. As the concentration of GTP was raised above 0.5 μM, the amount of GTPase activity for the reaction that contained FtsY(D441N) reached a plateau at about half the amount seen in the reaction that contained wild-type FtsY (Fig. 3B). One possible explanation for this difference would be that FtsY(D441N) function was inhibited by high concentrations of GTP. However, it was also possible that FtsY also hydrolyzed GTP. If this were the case, the contribution of FtsY(D441N) to the overall hydrolysis of nucleotide triphosphate would have escaped detection in this experiment because unlabeled XTP was used.

We tested the ability of FtsY(D441N) to hydrolyze [γ-^{32}P]XTP in the presence of Ffh-4.5*S* RNP and as a function of GTP concentration (Fig. 4). XTP hydrolysis was observed when GTP was added to the reaction, whereas no hydrolysis occurred in the absence of GTP (Fig. 4A). No XTP hydrolysis was observed in reactions that contained wild-type FtsY, nor, as expected, did FtsY(D441N) display any XTPase activity in the absence of Ffh-4.5*S* RNP (Fig. 4B). GDP did not stimulate the XTPase activity of FtsY(D441N) and inhibited the GTP-stimulated reaction (Fig. 4C). These results are thus similar to those observed above for FtsY(D441N) in the GTPase reaction. Thus, in the wild-type situation, Ffh and FtsY stimulate each other's GTPase activity and, moreover, to do so each protein must be in the GTP-bound state.

Ffh-4.5*S* RNP and FtsY could function either as GNRFs or as GAPs to stimulate GTP hydrolysis. We consider it unlikely that both proteins are GNRFs. First, it is difficult to envision how nucleotide exchange could be initiated during a cycle of Ffh-4.5*S* RNP–FtsY interaction if both proteins started out in the GDP-bound form. Second, the mechanism of GDP release that has been described for other well-characterized GNRFs involves stabilization of the nucleotide-free state of the GTPase, with which the GNRFs form stable complexes (*4*). In contrast, a stable Ffh-4.5*S* RNP–FtsY interaction requires the presence of nonhydrolyzable GTP analogs. Third, the affinity of Ffh for nucleotide is low [$K_m \approx 0.7$ μM (Fig. 1)] in comparison to that of other GTPases [for example, K_d of ET-Tu for GDP ≈ 2 nM (*1*)], which suggests that GDP release is not a rate-limiting step when purified components are assayed in vitro. The affinity of FtsY for nucleotides remains to be determined; however, the affinity of the α subunit of SRP receptor (the mammalian homolog of FtsY) for nucleotides is also low [$K_d \approx 10$ μM (*13*)].

We favor a model in which both GTPases act as GAPs for one another. The mechanism of hydrolysis would involve a concerted reaction in which both GTP molecules are hydrolyzed synchronously; if the GTP bound to one of the two GTPases was hydrolyzed first, this protein would no longer be activated and hence would be unable to stimulate GTP hydrolysis by its partner (*14*). This model implies a symmetry in the enzymology that parallels the strong similarity in the primary structures of the GTPase domains of Ffh and FtsY (*5*). Thus, the Ffh-FtsY interaction may have evolved from an originally homotypic interaction involving two identical ancestral components. Evolutionary divergence of the two interacting GTPases would have allowed them to develop affinities for different binding partners, thereby providing the means for linking these components together in a tightly controlled reaction (*15*). The finding that two GTPases can act as GTPase regulatory proteins for one another expands the repertoire of possible mechanisms by which GTPases control biological processes. Our results suggest how GTP is used during protein targeting. Ffh and FtsY, like eukaryotic SRP and SRP receptor, may promote interactions between the ribosome-nascent chain and the membrane-bound protein translocation apparatus (*7*, *16*). After proper matching of the ribosome and the translocation apparatus, concerted GTP hydrolysis by both components would allow Ffh and FtsY to be released from one another to enter another round of targeting.

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11. Abbreviations for the amino acid residues are as follows: D, Asp; K, Lys; N, Asn; and X, any amino acid.
12. An ~825–base pair Sac II–Eco RI fragment encompassing the GTP-binding domain of FtsY was subcloned into plasmid Blue Script SK+ (Stratagene). Site-directed mutagenesis [T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985); L. B. Evnin and C. S. Craik, *Ann. N.Y. Acad. Sci.* **542**, 61 (1988)] was used to introduce the Asp → Asn mutation corresponding to position 441 in the coding region of FtsY by means of the 20-mer oligonucleotide 5′-CGAAACTGAACGGCACGGCG-3′. This fragment was then introduced into a pGEX1 vector that contained FtsY fused to the COOH-terminus of GST, as described (*7*). The resulting mutant FtsY-GST fusion protein was then expressed and purified (*7*).
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14. We cannot, however, rule out more complicated alternative mechanisms. For example, the hydrolysis of only one GTP bound to either Ffh or FtsY might be sufficient to dissociate Ffh-4.5*S* RNP and FtsY during an interaction cycle, or FtsY may function as a GAP for Ffh while Ffh functions as a GNRF for FtsY (for which the affinity of GDP is unknown). The reverse of the latter scenario is very unlikely because of the low affinity of Ffh for nucleotide (Fig. 1).
15. This scenario additionally requires that after divergence, these GTPases no longer stimulate their own GTPase. In this regard, we note that the basal, FtsY-independent GTP hydrolysis rate of Ffh-4.5*S* RNP is strictly linear with enzyme concentration, with no detectable cooperativity (H. Wilhelm and P. Walter, unpublished data). Thus, this basal reaction does not seem to involve the cooperation of two Ffh molecules, but rather results from an intrinsic property of monomeric Ffh itself.
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TRAF2-Mediated Activation of NF-κB by TNF Receptor 2 and CD40

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TNF receptor–associated factor (TRAF) proteins are candidate signal transducers that associate with the cytoplasmic domains of members of the tumor necrosis factor (TNF) receptor superfamily. The role of TRAFs in the TNF-R2 and CD40 signal transduction pathways, which result in the activation of transcription factor NF-κB, was investigated. Overexpression of TRAF2, but not TRAF1 or TRAF3, was sufficient to induce NF-κB activation. A truncated derivative of TRAF2 lacking an amino-terminal RING finger domain was a dominant-negative inhibitor of NF-κB activation mediated by TNF-R2 and CD40. Thus, TRAF2 is a common mediator of TNF-R2 and CD40 signaling.

Cytokines trigger changes in gene expression by modifying the activity of otherwise latent transcription factors (*1*). TNF is an inducer of nuclear factor κB (NF-κB) (*2*), a homo- or heterodimer of members of the Rel family of transcriptional activators that control the expression of a variety of important cellular and viral genes (*3*, *4*). TNF initiates pleiotropic inflammatory and immunoregulatory responses by binding to two distinct cell surface receptors of ~55 kD (TNF-R1) and 75 kD (TNF-R2) (*5*). Both TNF receptors independently mediate NF-κB activation by TNF (*6*–*8*). Two putative effectors of TNF-R2 signaling have been identified—TRAF1 and TRAF2—that share a COOH-terminal homology region, the TRAF domain (*7*). TRAF2 homodimers as well as TRAF1:TRAF2 heterodimers can associate with a COOH-terminal region in the cytoplasmic domain of TNF-R2 that is required for signaling growth proliferation and NF-κB activation. A third TRAF domain protein, TRAF3 [also known as CD40bp, CRAF1, or LAP1 (*9*–*11*)], interacts with the cytoplasmic domain of CD40, another member of the TNF receptor superfamily (*12*). Because CD40 can also signal NF-κB activation (*13*), we investigated the potential role of TRAF proteins in this process.

In the yeast two-hybrid assay (*14*), TRAF3 self-associated, but it did not form heterotypic complexes with either TRAF1 or TRAF2 (Table 1). None of the three TRAFs associated with the cytoplasmic domain of either TNF-R1 or the Fas antigen (Table 1). Also, TRAF1 did not interact with CD40, nor did TRAF3 interact with TNF-R2. However, TRAF2 associated strongly with the cytoplasmic domains of both CD40 and TNF-R2 (Table 1) (*15*).

Induction of NF-κB activity by TNF has been demonstrated in numerous cell types, including human embryonic kidney 293 cells (*8*) (Fig. 1). In 293 cells, both TNF receptors are capable of signaling NF-κB activation. Because of a low level of endogenous TNF-R2 (*16*), only TNF-R1 mediates NF-κB activation in non-transfected 293 cells as indicated by stimulation with receptor-specific agonistic antibodies (Fig. 1). However, upon transfection TNF-R2 also signals NF-κB activation (below). To examine a functional role for TRAFs in NF-κB activation, we transiently transfected 293 cells with TRAF expression vectors (*17*) and performed electrophoretic mobility–shift assays (*18*). Expression of TRAF1 or TRAF3 did not result in induction of NF-κB DNA-binding activity. In contrast, TRAF2-expressing 293 cells contained activated NF-κB (Fig. 1). The major component of the active NF-κB complex was the p65:p50 heterodimer as indicated by supershift experiments (Fig. 1) (*18*).

To determine whether TRAF2 expression might activate an NF-κB–dependent reporter gene, we cotransfected an E-selectin–luciferase reporter construct (*19*) with the various TRAF expression vectors into 293 cells (*18*). TRAF2 expression potently activated the reporter gene, whereas expression of TRAF1 or TRAF3 had no effect (Fig. 2A). In all cases, reporter gene activity could be coinduced through the TNF-R1 pathway by the addition of TNF (Fig. 2A) (*20*). The observed reporter gene induction was dependent on NF-κB activation because TRAF2 expression failed to activate a control reporter construct in which the NF-κB sites in the E-selectin promotor were mutated (*21*). Thus, overexpression of TRAF2, but not of TRAF1 or TRAF3, is sufficient to initiate NF-κB activation in 293 cells. This observation is consistent with an activation mechanism in which TRAF2 clustering induced by its overexpression is similar to that induced by ligand-triggered receptor aggregation, thereby activating the NF-κB signaling pathway independently of TRAF2 association with TNF-R2.

TRAF2 and TRAF3 contain NH_2-terminal RING finger motifs that are not re-

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