

Chaperone release and unfolding of substrates in type III secretion

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Type III protein secretion systems are essential virulence factors of many bacteria pathogenic to humans, animals and plants¹. These systems mediate the transfer of bacterial virulence proteins directly into the host cell cytoplasm. Proteins are thought to travel this pathway in a largely unfolded manner, and a family of customized cytoplasmic chaperones, which specifically bind cognate secreted proteins, are essential for secretion. Here we show that InvC, an ATPase associated with a *Salmonella enterica* type III secretion system², has a critical function in substrate recognition. Furthermore, InvC induces chaperone release from and unfolding of the cognate secreted protein in an ATP-dependent manner. Our results show a similarity between the mechanisms of substrate recognition by type III protein secretion systems and AAA+ ATPase disassembly machines.

Containing more than 20 proteins, type III secretion systems (TTSSs) are among the most complex protein secretion machines known^{1,3}. A central component of TTSSs is an envelope-associated organelle known as the needle complex⁴. This organelle is traversed by a central channel 28 Å in diameter, which is thought to serve as a conduit for the proteins transiting this secretion pathway⁵. Other essential components of TTSSs include an ATPase and a family of customized chaperones that specifically bind a ~100-amino-acid domain at the amino terminus of their cognate secretion substrates⁶. This domain wraps around homodimer chaperone pairs and is maintained in a non-globular conformation that retains significant secondary structure^{7,8}. After secretion, the chaperones must release their cognate substrate because they remain within the bacterial cytoplasm. In addition to the chaperone-binding domain, specific elements required for secretion have been also localized N-terminal of this domain of the secreted protein^{9–11}.

InvC is the ATPase associated with the TTSS encoded by *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) within its pathogenicity island 1 (ref. 2), which mediates bacterial entry into mammalian cells and is essential for the virulence of this bacterial pathogen¹². Like other members of the TTSS-associated ATPase protein family¹³, InvC has extensive primary amino acid sequence similarity to the β-subunit of F₀F₁-ATPases. Although these ATPases are essential for type III secretion (TTS), their specific role in this process is unknown. Their peripheral association to the cytoplasmic side of the bacterial membrane coupled to their ability to form hexameric rings^{14–16}, indicated the possibility that these ATPases might provide a link between the secretion substrates and the TTS machinery. We therefore investigated the potential role of InvC in TTS substrate recognition by examining its interaction with the type III secreted protein SptP bound to its cognate chaperone SicP. SptP is a SPI-1 TTS effector protein that poses two enzymatic activities: a Rho-family GTPase-activating protein (GAP) activity located immediately adjacent to the chaperone-binding domain, and a protein tyrosine phosphatase located at the carboxy terminus^{7,17,18}

(Fig. 1a). The full-length SptP (SptP^{1–543}) or its chaperone-binding domain (SptP^{1–158}) bound to its glutathione S-transferase (GST)-tagged cognate chaperone SicP were purified to homogeneity and their interaction with purified InvC was probed by affinity chromatography. The GST-SicP-SptP^{1–543} or GST-SicP-SptP^{1–158} complex specifically interacted with InvC but not with the irrelevant protein CdtA (Fig. 1b, d). The interaction occurred both in the presence of ATP and in the presence of ATP-γS, indicating that the ability of InvC to hydrolyze ATP is not essential for substrate recognition (Fig. 1b). Both the monomeric and hexameric forms of InvC were able to interact with the SicP-SptP complex, although the latter appeared to do so more efficiently (Fig. 1c). InvC also interacted with the GST-tagged chaperone alone but did not interact with the effector domains of SptP (SptP^{161–543}) (Fig. 1d). These results indicate that InvC is able to recognize both the SicP chaperone and the chaperone/secreted protein complex, and that its multimeric form appears to be more efficient at substrate recognition. Another TTSS-associated ATPase has been also reported to bind TTSS chaperones¹⁹.

We have previously isolated loss-of-function mutations in InvC, which allowed the identification of specific residues important for its catalytic activity, membrane association, and oligomerization¹⁶. Mutations that affected either the membrane-binding (InvC^{V28M}) or catalytic activities (InvC^{K165E}) did not affect the ability of InvC to bind the SicP-SptP complex (Fig. 1e). However, a loss-of-function mutant (InvC^{L376P}) that retained its catalytic, membrane and oligomerization activities, did not bind the SicP-SptP complex (Fig. 1e). This mutant is located at the C terminus of InvC within a domain that has been predicted to face the cytoplasm¹⁶. Therefore, this mutant most likely defines the InvC substrate-binding domain. Taken together, these results indicate that InvC interacts with the chaperone and chaperone/effector protein complex through a domain located at its C terminus and therefore must play a critical role in substrate recognition by the TTS machine.

Immediately before secretion, TTSS-associated chaperones must dissociate from the chaperone/effector complex. We investigated whether the interaction of the SicP-SptP complex with InvC resulted in the release of the chaperone from its cognate substrate. Wild-type InvC or the catalytically inactive mutant InvC^{K165E} were incubated with purified GST-SicP-SptP^{1–158} complex in the presence of ATP or ATP-γS and the dissociation of the complex was examined with an affinity chromatography assay. In the presence of wild-type InvC and ATP, SptP^{1–158} was released from its association with SicP (Fig. 2). Equivalent results were obtained with the GST-SicP-SptP^{1–543} complex (data not shown). In contrast, the release of SptP^{1–158} was not observed when the complex was incubated with the catalytically inactive InvC^{K165E} or in the presence of ATP-γS (Fig. 2). These results indicate that InvC is capable of removing SptP from its chaperoned state and that this activity requires ATP hydrolysis.

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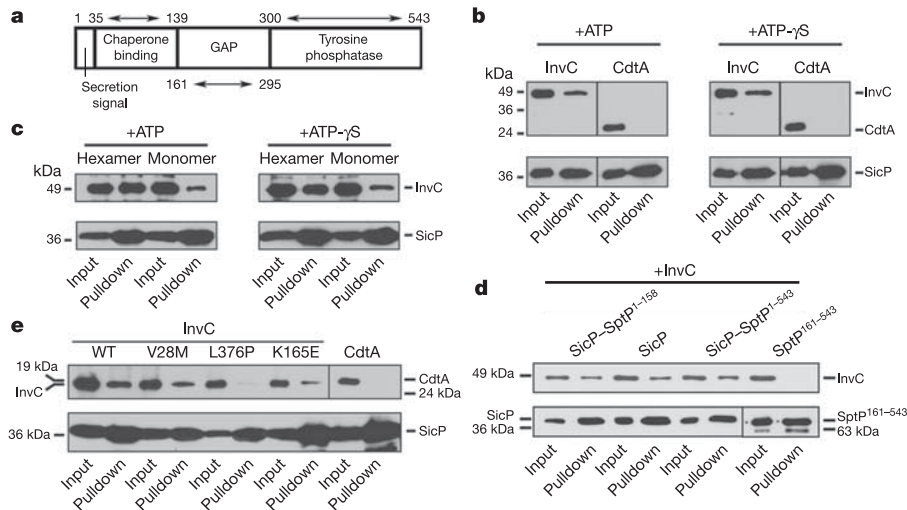


Figure 1 | The ATPase InvC interacts with TTS substrates. **a**, Diagram of the domain organization of the type III secreted protein SptP. **b**, Interaction of InvC with the SicP–SptP complex. Top, purified GST–SicP–SptP^{1–158} complex was incubated with InvC or CdtA (negative control) in the presence of ATP or ATP- γ S. The presence of InvC or CdtA in the input sample (input) or bound to the GST–SicP–SptP^{1–158} complex (pulldown) was examined by western immunoblotting with an anti-His-tag monoclonal antibody. Bottom, the total amount of GST–SicP was determined by western

immunoblotting with an anti-GST antibody. **c**, The interaction GST–SicP–SptP^{1–158} with monomeric or hexameric InvC in the presence of ATP or ATP- γ S was examined as described above. **d**, The interaction of InvC with GST–SicP, GST–SicP–SptP^{1–158}, GST–SicP–SptP^{1–543} or GST–SptP^{161–543} was examined as indicated above. **e**, The interaction of purified InvC mutants (as indicated) with the GST–SicP–SptP complex was examined as described above. WT, wild type.

The size constraints imposed by the diameter of the central channel of the TTSS-associated needle complex indicates that proteins travelling the TTS pathway must do so in a largely unfolded manner⁵. Although the TTSS-associated chaperones maintain the binding domains of their cognate secreted proteins in a non-globular state⁷, the enzymatic domains of the secreted proteins remain folded because they exhibit full activity²⁰. This observation indicated that unfolding of these domains must occur before secretion. We therefore examined whether InvC could mediate the unfolding of SptP upon its release from its cognate chaperone SicP. We first probed conformational changes in SptP by a protease susceptibility assay with the use of a monoclonal antibody that recognizes the C-terminal domain of SptP. Addition of InvC to the SicP–SptP complex in the presence of ATP resulted in a significant increase in the susceptibility of SptP to protease (Fig. 3a). In contrast, addition of InvC in the presence of ATP- γ S or addition of the catalytically inactive mutant InvC^{K165E} did not increase the susceptibility of SptP to protease (Fig. 3a). These results are consistent with the hypothesis that InvC induces significant conformational changes in SptP and that this function requires its catalytic activity. We further explored this issue with a different assay. We reasoned that if InvC induces global unfolding of SptP, these changes should significantly affect its enzymatic activity. We therefore probed the conformational changes of SptP during its interaction with InvC by assaying its tyrosine phosphatase activity. The tyrosine phosphatase domain is the most distant from the chaperone-binding region, from which it is separated by the GAP domain (Fig. 1a). Addition of wild-type InvC to the SicP–SptP complex resulted in a drastic decrease in the phosphatase activity of SptP (Fig. 3b). In contrast, addition of the catalytically inactive InvC^{K165E} mutant had no effect on the phosphatase activity of SptP (Fig. 3b). Furthermore, InvC had no effect on the activity of SptP^{161–543}, a mutant that lacks the chaperone-binding domain (Fig. 3b), indicating that this domain is required for InvC to catalyse the unfolding of SptP. We also tested the unfolding of SptP by examining its ‘transfer’ to a GroEL ‘trap’ mutant (GroEL^{D87K}), which captures non-native proteins but is unable to release them²¹. We incubated the SicP–SptP^{1–543} complex with InvC or InvC^{K165E} in the presence of GroEL^{D87K} and subjected the mixture to gel-filtration chromatography. Addition of InvC, but not its catalytic mutant

InvC^{K165E}, significantly changed the migration of SptP (Supplementary Fig. 1). Instead of eluting at a position corresponding to the size of the SicP–SptP^{1–543} complex, SptP migrated with a broader profile and a significant proportion of the input migrated at the position of the ‘trap’, indicating the binding of non-native forms of SptP to GroEL^{D87K} (Supplementary Fig. 1). Addition of GroEL to the complex in the presence of InvC^{K165E} did not result in any change in the mobility of SptP, indicating that the GroEL trap alone does not affect the folding of SptP (Supplementary Fig. 1). Taken together, these results indicate that InvC can not only remove the chaperone but can also induce the global unfolding of SptP.

Secretion and translocation domains of type III secreted proteins are able to mediate the secretion of heterologous proteins²². However, secretion of some proteins is inefficient or does not occur at all²³, indicating that certain domains are not permissive for TTS because they cannot be unfolded by the ATPase. To test this hypothesis we fused the TTS targeting signals of SptP to the green fluorescent protein (GFP), which is known to have a very compact and stable fold. The GST–SicP–SptP^{1–158}–GFP protein complex was isolated and the ability of InvC to unfold the GFP domain was then examined

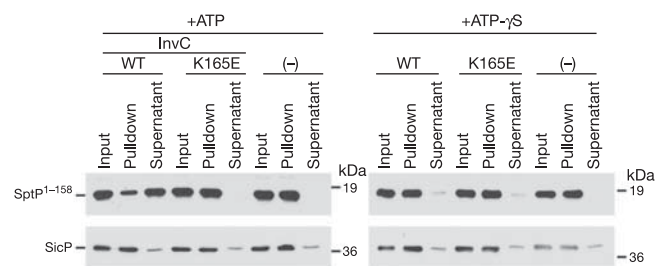


Figure 2 | InvC disassembles the chaperone/secreted protein complex. Purified InvC and the catalytic InvC^{K165E} mutant were added to purified GST–SicP–SptP^{1–158} complex in the presence of ATP or ATP- γ S. The presence of released SptP^{1–158} (supernatant) or in association with GST–SicP (pulldown) was assayed by a GST-pulldown assay and western immunoblotting with a monoclonal antibody directed against the N terminus of SptP or the GST tag present in SicP.

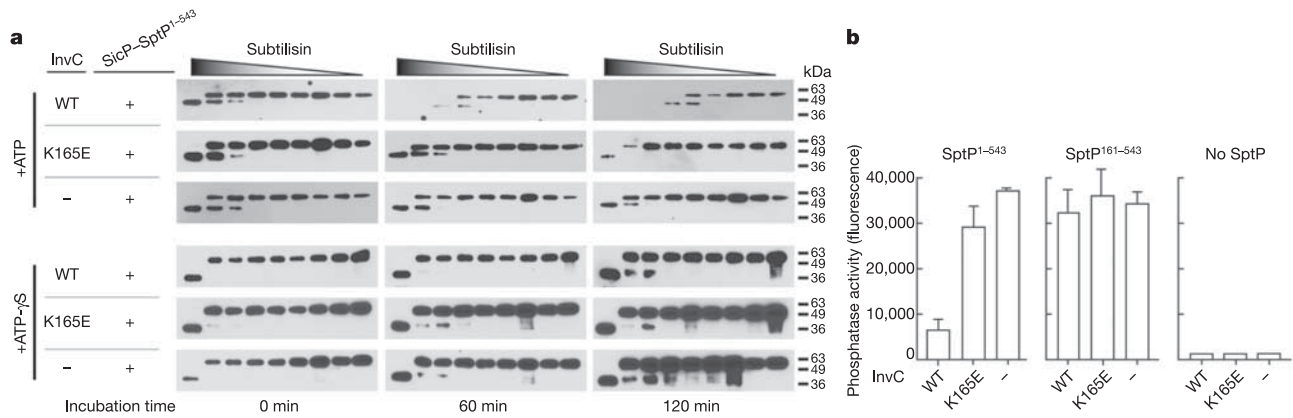


Figure 3 | InvC induces the unfolding of SptP. **a**, Purified SicP–SptP^{1–543} complex was incubated with wild-type (WT) InvC or its catalytic mutant InvC^{K165E} in the presence of ATP or ATP-γS for the indicated durations. Decreasing amounts of subtilisin were then added and the presence of SptP in the different samples was analysed by western immunoblotting with an

antibody directed against its C terminus. **b**, WT InvC or its catalytic mutant InvC^{K165E} was added to the SicP–SptP complex or SptP^{161–543}, and the tyrosine phosphatase activity was measured as indicated in Methods. Results are means ± s.d. for three independent measurements.

by monitoring the GFP fluorescence. Addition of InvC resulted in a release of SicP (Fig. 4a) but had no effect on the fluorescence activity of the SptP^{1–158}–GFP fusion protein (Fig. 4b), indicating that InvC was unable to unfold the tightly packed GFP domain. If unfolding is a requirement for type III protein secretion, the inability of InvC to unfold GFP should prevent its secretion through the TTS pathway. We tested this hypothesis by examining the secretion of SptP–GFP into culture supernatants of *S. typhimurium*. SptP–GFP was not

secreted into the culture supernatant of *S. typhimurium*, indicating that the inability of InvC to unfold the SptP–GFP chimera renders this protein non-competent for secretion (Fig. 4c). In contrast, fusion of the targeting signals of SptP to PhoA, which is thermodynamically less stable²⁴, resulted in a chimera that was efficiently unfolded by InvC (Fig. 4d) and was efficiently secreted by the *S. typhimurium* TTS pathway (Fig. 4e). These results indicate that proteins that are destined to be secreted by the type III pathway must

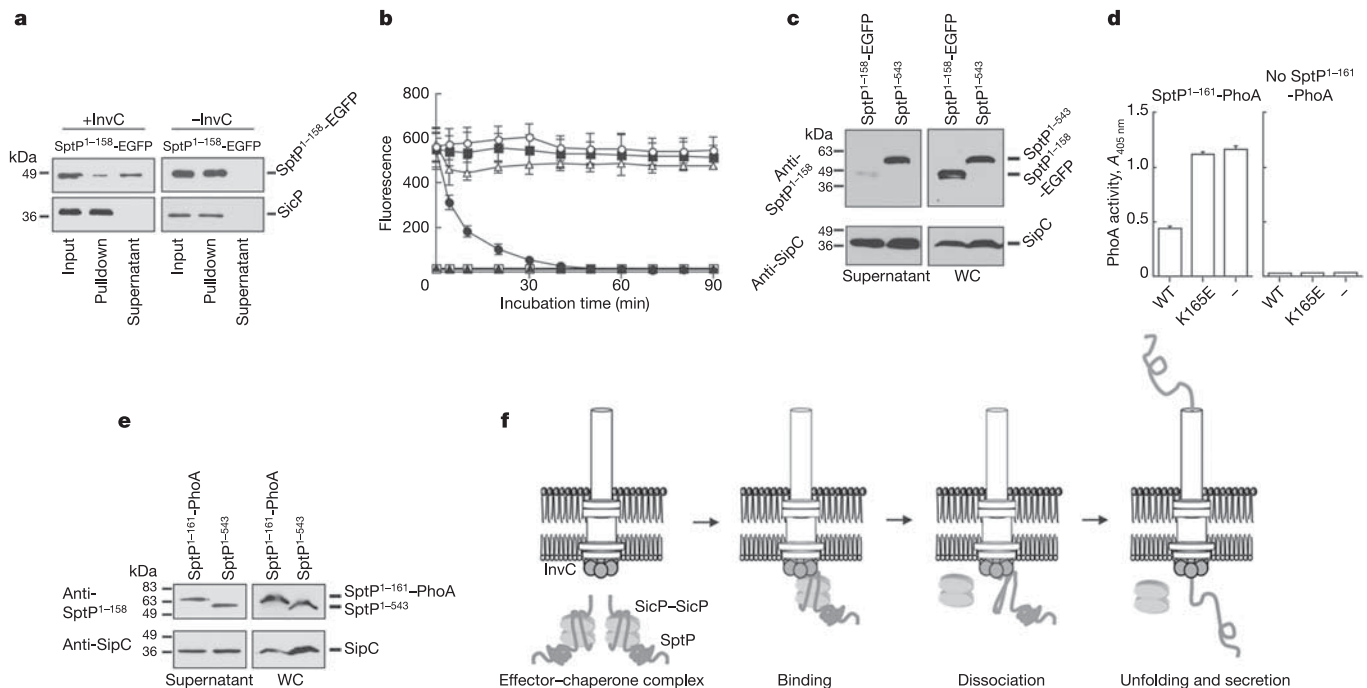


Figure 4 | Substrate unfolding is required for TTS. **a**, InvC was added to the SptP^{1–158}–GFP protein complex in the presence of ATP; the release of SptP^{1–158}–GFP was assayed as described in Fig. 2. **b**, InvC or its catalytic mutant InvC^{K165E} was added to purified SicP–SptP^{1–158}–GFP complex and the GFP fluorescence ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 538$ nm) of the samples was measured over time. Results are means ± s.d. for three independent measurements. Open circles, InvC^{K165E} plus SicP–SptP^{1–158}–EGFP; filled squares, SicP–SptP^{1–158}–EGFP; open triangles, InvC plus SicP–SptP^{1–158}–EGFP; filled circles, SicP–SptP^{1–158}–EGFP plus 6 M guanidinium chloride; open squares, InvC; filled triangles, InvC^{K165E}; open diamonds, buffer. **c**, Top, culture supernatants and whole-cell lysates

(WC) from *S. typhimurium* SB1404 expressing SicP along with wild-type SptP or SptP^{1–158}–GFP were separated by SDS–PAGE and analysed for the presence of the different proteins by western immunoblot analysis. Bottom, as a control, blots were re-probed to examine the presence of the type III secreted protein SipC. **d**, InvC or its catalytic mutant InvC^{K165E} was added to the SicP–SptP^{1–161}–PhoA complex and the phosphatase activity was measured as indicated in Methods. Results are means ± s.d. for three independent measurements. **e**, The secretion of SptP^{1–161}–PhoA into culture supernatants of *S. typhimurium* SB1404 was examined as indicated above. **f**, Model for TTS (see the text for details).

be 'primed' for the ATPase-catalysed unfolding. Indeed, some features of the crystal structure of the enzymatic domains of SptP are consistent with this hypothesis. For example, the GAP domain region immediately adjacent to the chaperone-binding domain seemed highly disordered¹⁸. In addition, the better-ordered portion of the GAP domain stabilizes an extended-loop region at the N terminus of the PTP domain. It is therefore possible that, once the GAP domain has been unravelled, the loss of support for this extended-loop structure could facilitate the unravelling of the entire PTP domain.

Our results show a central role for the TTSS-associated ATPases in hitherto poorly understood steps of protein secretion, such as substrate recognition and chaperone release from, and unfolding of, type III secreted proteins (Fig. 4f). Our results also revealed remarkable parallels between InvC and the function of triple AAA+ ATPases, a family of proteins involved in a variety of biological processes^{25,26}. Although the TTSS-associated ATPases have not previously been considered to be members of this protein family²⁷, the ability of InvC to organize in hexameric rings, to disassemble protein complexes and to recognize and unfold protein substrates closely resembles equivalent activities of AAA+ ATPase disassembly machines^{25,28}. The similarities seem to extend to essential features of the substrate recognition process, because TTSSs and AAA+ machines recognize a poorly conserved set of unstructured short peptide signals located at the N terminus of the target proteins. TTS of virulence factors and the translocation of substrates into triple AAA+ machines might therefore have a common evolutionary origin and might consequently share mechanistic features.

METHODS

Bacterial strains. *Escherichia coli* XL-1 Blue (Invitrogen) was used for DNA manipulations and *E. coli* BL-21 (DE3) was used for the expression of recombinant proteins. The *Salmonella enterica* serovar Typhimurium SB1404, which carries a $\Delta sicP-sptP$ deletion, was constructed by allelic exchange as described previously²⁹.

Plasmids. Bicistronic plasmids encoding GST-SicP-SptP¹⁻¹⁵⁸, GST-SicP-SptP¹⁻⁵⁴³, GST-SicP-SptP¹⁻¹⁵⁸-EGFP and GST-SicP-SptP¹⁻¹⁶¹-PhoA³⁵⁻⁴⁷² were constructed by standard recombinant DNA techniques with the expression plasmid pGEX-KG as a backbone. Plasmids expressing GST-SptP¹⁶¹⁻⁵⁴³ (ref. 18), polyhistidine-tagged InvC and its mutant forms¹⁶, or polyhistidine-tagged CdtA, a subunit of the cytolethal distending toxin³⁰, have been described previously.

Purification of recombinant proteins. Recombinant polyhistidine-tagged monomeric and hexameric InvC were purified as described elsewhere¹⁶. Monomeric and hexameric forms of purified InvC were separated by gel-filtration chromatography as described previously¹⁶. GST-SicP in complex with SptP¹⁻¹⁵⁸, SptP¹⁻⁵⁴³ or the chimaeric protein SptP¹⁻¹⁵⁸-EGFP or SptP¹⁻¹⁶¹-PhoA³⁵⁻⁴⁷² was purified from *E. coli* carrying the respective bicistronic expression plasmids as described previously⁷.

Affinity chromatography binding assay. Purified GST-SicP-SptP¹⁻¹⁵⁸ complex was incubated with purified polyhistidine-tagged wild-type InvC or its mutants (InvC^{V28M}, InvC^{K165E} or InvC^{L376P}) in a binding buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 0.05% Tween 20, 0.001% BSA) in the presence of 100 μ M ATP or ATP- γ S for 30 min at 4 °C. Glutathione-Sepharose 4 FastFlow beads were added to the mixtures and incubated for 1 h at 4 °C. After the beads had been washed to remove unbound proteins, the samples were subjected to SDS-PAGE. Proteins were detected by western blotting with monoclonal antibodies directed against the different relevant proteins.

Chaperone release assay. Purified SicP-GST-SptP¹⁻¹⁵⁸ complex was absorbed with glutathione-Sepharose 4B FastFlow at 37 °C for 1 h. After the beads had been washed to remove unbound complex, wild-type InvC or InvC^{K165E} was added to the beads in the releasing buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 0.05% Tween 20, 0.001% BSA) in the presence of 100 μ M ATP or ATP- γ S. After gentle rocking at 4 °C for 1 h, samples were separated into supernatant and beads by centrifugation. Supernatant was precipitated with trichloroacetic acid. The resulting samples were subjected to SDS-PAGE, and proteins were detected by western immunoblotting.

Protease susceptibility assay. Purified SicP-SptP¹⁻⁵⁴³ complex was incubated with wild-type InvC or InvC^{K165E} in reaction buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 0.05% Tween 20, 0.001% BSA, 100 μ M ATP or 100 μ M ATP- γ S, as indicated) at 37 °C. At several time points, different amounts

of subtilisin (10⁻⁵ to 10⁻⁸ U) were added to the samples and incubated on ice for 30 min. To stop the protease reaction, 1 mM PMSF was added to each tube. All the samples were subjected to SDS-PAGE and proteins were detected by western blotting.

Protein tyrosine and alkaline phosphatase assays. The purified SicP-SptP¹⁻⁵⁴³ complex or SptP¹⁶¹⁻⁵⁴³ was mixed with the purified hexameric form of wild-type InvC or the catalytic InvC^{K165E} mutant at a molar ratio of 1:10 and incubated at 37 °C for 30 min; the tyrosine phosphatase activity was measured with a RediPlate 96 EnzChek tyrosine phosphatase assay kit (Molecular Probes) in accordance with the manufacturer's recommendations. The purified SicP-SptP¹⁻¹⁵⁸-PhoA³⁵⁻⁴⁷² was mixed with the purified hexameric form of wild-type InvC or the catalytic InvC^{K165E} mutant as described above and the phosphatase activity was measured with the substrate *p*-nitrophenyl phosphate with the use of standard procedures.

EGFP fluorescence assay. Purified GST-SicP-SptP¹⁻¹⁵⁸-EGFP complex was incubated with wild-type InvC or the catalytic InvC^{K165E} mutant in assay buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 100 μ M ATP, 0.05% Tween 20, 0.001% BSA) at 37 °C for different durations and the fluorescence (excitation wavelength 488 nm, emission wavelength 538 nm) in the samples was measured in a fluorimeter (Molecular Devices).

Received 5 April; accepted 1 July 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank A. Horwich and G. Farr for providing us with GroEL^{D87K}, S.-H. Lee for plasmid constructs, and members of the Galán laboratory for critical reading of the manuscript. This work was supported by a Public Health Service Grant.

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