This is a repository copy of Systematic identification of conserved bacterial c-di-AMP receptor proteins.

White Rose Research Online URL for this paper:
http://eprints.whiterose.ac.uk/96337/

Version: Accepted Version

Article:

https://doi.org/10.1073/pnas.1300595110

Reuse
Unless indicated otherwise, fulltext items are protected by copyright with all rights reserved. The copyright exception in section 29 of the Copyright, Designs and Patents Act 1988 allows the making of a single copy solely for the purpose of non-commercial research or private study within the limits of fair dealing. The publisher or other rights-holder may allow further reproduction and re-use of this version - refer to the White Rose Research Online record for this item. Where records identify the publisher as the copyright holder, users can verify any specific terms of use on the publisher’s website.

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.
Systematic identification of conserved bacterial c-di-AMP receptor proteins

Rebecca M. Corrigan*, Ivan Campeotto†, Tharskha Jeganathan*, Kevin G. Roelofs*, Vincent T. Lee‡ and Angelika Gründling*‡

* Section of Microbiology and MRC Centre for Molecular Bacteriology and Infection, Imperial College London, London, SW7 2AZ, UK. † Department of Cell Biology and Molecular Genetics, and Maryland Pathogen Research Institute, University of Maryland, College Park, MD 20742, USA.

Submitted to Proceedings of the National Academy of Sciences of the United States of America

Nucleotide signaling molecules are important messengers in key pathways that allow cellular responses to changing environments. Canonical secondary signaling molecules act through specific receptor proteins by direct binding to alter their activity. Cyclic diadenosine monophosphate (c-di-AMP) is an essential signaling molecule in bacteria that has only recently been discovered. Here, we report on the identification of four Staphylococcus aureus c-di-AMP receptor proteins that are also widely distributed among other bacteria. Using an affinity pull-down assay, we identified the potassium transporter-gating component KtrA as c-di-AMP receptor protein and it was further shown that this protein, together with c-di-AMP, enables S. aureus to grow in low potassium conditions. We defined the c-di-AMP binding activity within KtrA to the RCK_C (regulator of conductance of K+) domain. This domain is also found in a second S. aureus protein, CpaA, which as we show here also directly binds c-di-AMP. Since RCK_C domains are found in proteinaceous channels, transporters and antiporters from all kingdoms of life, these findings have broad implications for the regulation of different pathways through nucleotide-dependent signaling. Using a genome-wide nucleotide protein interaction screen, we further identified the histidine kinase protein KdpD that in many bacteria is also involved in the regulation of potassium transport and a PII-like signal transduction protein, which we renamed PstA, as c-di-AMP binding proteins. With the identification of these widely distributed c-di-AMP receptor proteins we link the c-di-AMP signaling network to a central metabolic process in bacteria.

c-di-AMP | receptor protein | Staphylococcus

Introduction. Nucleotide signaling molecules control fundamental processes in all forms of life. There is now a large body of evidence linking nucleotides such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and guanosine tetra-(ppGpp) and pentaphosphate (pppGpp) to the control of fundamental metabolic pathways and stress response processes in eukaryotic and prokaryotic cells (1-3). Cyclic dinucleotides in particular have recently gained increased attention with the identification of novel nucleotides such as cyclic diadenosine monophosphate (c-di-AMP) and the hybrid cAMP-GMP molecule in bacterial cells (4-6), as well as the discovery that cyclic dinucleotides are also produced by eukaryotic cells (7-9). The dinucleotide cyclic diadenosine monophosphate (c-di-GMP) and the molecular mechanisms by which it controls cellular processes has been well characterized and it is now recognized as a central regulator in bacterial cells that controls the switch from free-living planktonic to a sessile biofilm-associated lifestyles. In pathogenic organisms this is often linked to colonization of the human host and virulence (10). On the other hand, the function and the pathways controlled by the novel signaling nucleotide c-di-AMP are less clear, largely due to a gap in our knowledge of specific receptor proteins.

Many Gram-positive bacteria, including the important human pathogens Staphylococcus aureus (11), Streptococcus pyogenes (12), Listeria monocytogenes (5) and Mycobacterium tuberculosis (13) produce c-di-AMP and it is likely that c-di-AMP is also synthesized by several Gram-negative bacteria and a subset of archaea (14). c-di-AMP is synthesized by DisA_N domain-containing proteins DacA, DisA and YojJ and degraded by the DHII/ DHHA1 domain-containing phosphodiesterase enzyme GdpP (4, 5, 11, 15-18). A variety of different phenotypes have been linked to altered c-di-AMP levels; an increase in c-di-AMP levels correlates with increased acid resistance (16, 19) and altered antibiotic resistance including an increase in methicillin resistance in S. aureus (11, 18, 20). Most notable, however, are the findings that L. monocytogenes (5) and Bacillus subtilis (18) cannot grow in the absence of c-di-AMP, showing that in contrast to other signaling nucleotides, c-di-AMP controls essential cellular pathways. The molecular basis for this is currently not known, though it is assumed that, similar to other signalling molecules, c-di-AMP interacts with a specific set of target proteins and upon binding alters their activity or function. Currently only one bacterial c-di-AMP receptor protein, the TetR-type transcription factor DarR, has been identified in Mycobacterium smegmatis (21). However, the absence of close DarR homologs in many organisms that likely produce c-di-AMP implies that additional c-di-AMP target proteins must exist.

In this study we have identified KtrA as c-di-AMP target protein by using an affinity pull down assay. KtrA is a member of the widely distributed RCK (regulator of conductance of K+) protein family, known to be involved in the gating of ion channels. Here we show that KtrA is required for the growth of S. aureus under potassium limiting conditions. Through subsequent binding studies we show that c-di-AMP specifically interacts with the C-terminal RCK_C domain of KtrA. A second S. aureus RCK_C domain-containing protein CpaA, a predicted cation/proton antiporter, was subsequently identified bioinformatically and its interaction with c-di-AMP confirmed experimentally. Lastly, using a genome wide interaction screen, we identified the PII-like signal transduction protein PstA and the histidine kinase KdpD as additional c-di-AMP binding proteins. With the identification of these four novel and widely distributed c-di-AMP binding proteins we provide a link between c-di-AMP and a fundamental cellular process in bacteria, namely ion transport.

Results

Identification of the c-di-AMP target protein KtrA. To identify c-di-AMP receptor proteins, we performed an affinity pull down
assay using c-di-AMP-coupled magnetic beads and protein extracts derived from the *S. aureus* strain LAC*. This strain is an erythromycin sensitive derivative of the clinically relevant community-acquired methicillin resistant USA300 strain LAC. One protein band was enriched in samples obtained from c-di-AMP-coupled beads (Fig. 1A) and identified by mass spectrometry as *S. aureus* protein SAUSA300_0988 (KtrAS). (c) Illustration of Ktr-type potassium transport systems, which are composed of a KtrB-type membrane component and a cytoplasmic KtrA-type gating component. (C) Schematic representation of the KtrAS protein structure with the RCK_N domain (amino acids 4-126) indicated in blue and RCK_C domain (amino acids 135-219) shown in orange. The RCK_N domain of the B. subtilis KtrA homolog is known to bind to nucleotides including ATP, ADP, NAD* and NADH.

![Figure 1](image_url)

**Fig. 1.** Identification of *S. aureus* KtrAS as a potential c-di-AMP binding protein. (A) Silver polyacrylamide gel of cellular lysate from KtrAS protein retained on c-di-AMP-coupled (+) or uncoupled (-) beads. The protein band enriched in the c-di-AMP lane (indicated with an asterisk) was identified by mass spectrometry as *S. aureus* protein SAUSA300_0988 (KtrAS). (B) Illustration of Ktr-type potassium transport systems, which are composed of a KtrB-type membrane component and a cytoplasmic KtrA-type gating component. (C) Schematic representation of the KtrAS protein structure with the RCK_N domain (amino acids 4-126) indicated in blue and RCK_C domain (amino acids 135-219) shown in orange. The RCK_N domain of the B. subtilis KtrA homolog is known to bind to nucleotides including ATP, ADP, NAD* and NADH.

**KtrA is important for the growth of *S. aureus* in low potassium.** To investigate the involvement of KtrAS and c-di-AMP in the growth of *S. aureus* in low potassium conditions, the growth of ktrA and gdpP mutant strains was compared to that of the wildtype LAC* strain. The gdpP mutant strain has 15-fold higher levels of intracellular c-di-AMP (11) and therefore KtrA should be in the nucleotide-bound state under these conditions. Since potassium uptake is especially important during osmotic stress, the different *S. aureus* strains were grown on chemically defined medium (CDM) plates containing 0.75 M NaCl. Under these stress conditions, a two to three log growth defect was observed for both the ktrA and gdpP mutant strains, which could be complemented either by the addition of potassium or by the introduction of a functional copy of ktrA or gdpP, respectively (Fig. 3A and 3B). The ktrA mutant was also hyper-sensitive to the potassium ionophore nigericin, which causes an exchange of intracellular potassium bound to the RCK_C domain in the mM range for extracellular H+. (Fig. 3C). The hypersensitivity to nigericin could again be rescued by the addition of 250 mM potassium or by genetic complementation (Fig. 3C and 3D). Similarly a ktrA mutant strain in the methicillin sensitive *S. aureus* strain background Newman was also more sensitive to nigericin and did not grow as well as the wild-type strain under the osmotic stress conditions unless potassium was added (Fig. 5E). These results suggest a function for KtrAS in potassium uptake in *S. aureus* strains and that c-di-AMP binding to KtrAS might inactivate...
The identification of the RCK domain-containing protein.

**c-di-AMP interacts with CpaA, a second *S. aureus* RCK,C domain-containing protein.** The identification of the RCK,C domain as a c-di-AMP interacting domain allows the bioinformatic prediction of other receptor proteins based on the presence of an RCK,C domain. In this manner we discovered the protein SAUSA300-134-220 (RCK,C,N) or His-KtrA, which was determined from the curve as previously described (27). (C) DRaCALAs with purified His-KtrA,C domain and c-di-AMP was tested by performing DRaCALAs with purified His-KtrA,C domain.

**Footline Author PNAS**

**Fig. 2.** Characterization of the c-di-AMP / KtrA,C interaction by DRaCALA. (A) Schematic representation of the differential radial capillary action of ligand assay (DRaCALA) to study c-di-AMP protein interactions. (B) Binding curve and } K_{d} determination for c-di-AMP and purified His-KtrA,C. } K_{d} values were determined from the curve as previously described (27). (C) DRaCALAs with purified His-KtrA,C domain and c-di-AMP and an excess of cold competitor nucleotide as indicated above each spot. (D) DRaCALAs with purified His-KtrA,C domain and c-di-AMP and an excess of cold competitor nucleotide as indicated above each spot.

**Fig. 3.** Effect of potassium on growth of wildtype (WT), ktrA and gdpP *S. aureus* strains. (A) and (B), The indicated *S. aureus* strains were grown overnight in chemically defined medium (CDM) containing 2.5 mM KCl. Next day serial dilutions of washed cells were spotted onto CDM agar plates containing 0.75 M NaCl and containing either 0 mM or 2.5 mM potassium. (C) and (D), Nigericin sensitivity curves of WT, ktrA mutant complemented *S. aureus* strains. The different strains were grown in 96-well plates in CDM medium supplemented with 2.5 mM or 250 mM potassium and nigericin at the indicated concentration. OD_{600} readings were determined after 24 h growth and plotted as % growth compared to the growth in the absence of nigericin. Experiments were repeated a minimum of 5 times. When grown in 2.5 mM KCl the ktrA mutant consistently showed a two-fold reduced MIC for each experiment. The MIC for all the strains varied between experiments from 0.1-0.8 µM for the wildtype and complemented strain and 0.05-0.4 µM for the mutant strains.
A. S. aureus CpaA

<table>
<thead>
<tr>
<th>Na+/K+</th>
<th>H+</th>
</tr>
</thead>
<tbody>
<tr>
<td>402</td>
<td>516</td>
</tr>
<tr>
<td>531</td>
<td>612</td>
</tr>
<tr>
<td>614</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Identification of CpaA as an additional c-di-AMP target protein (A) Schematic representation of the predicted K+ or Na+ antporter CpaA (SAUSA300,0911), containing an N-terminal transmembrane (yellow) and cytoplasmically located RCK_N (blue) and RCK_C (orange) domains. (B) DRaCALAs with 32P-labeled c-di-AMP and E. coli extracts prepared from the vector control strain (pET28b) or strains overproducing His-CpaA_N402-614 (RCK_N and RCK_C) or His-CpaA_C513-614 (RCK_C). Cold c-di-AMP was added as a competitor where indicated.

Discussion

Since the discovery of c-di-AMP, it has been speculated that this nucleotide binds to proteins to regulate their function. In this study we identified four c-di-AMP receptor proteins, namely KtrA, CpaA, KdpD and PstA by using an affinity pull down assay, bioinformatics analysis and a genome-wide protein-nucleotide interaction screen (Figs. 1, 4 and 5). With the identification of three proteins (KtrA, CpaA, and KdpD) that have been implicated in potassium transport in other bacteria, we have linked c-di-AMP signaling to potassium transport in S. aureus. Interestingly, this distinguishes c-di-AMP from c-di-GMP, which regulates multiple cellular processes that help bacteria to transition between different lifestyles, such as extracellular carbohydrate and adhesion production, motility and biofilm formation. The link between c-di-AMP and the ion transport may explain why c-di-AMP, in contrast to other related signaling nucleotides, is essential for growth in bacterial species. Individually ktrA, cpaA, psta and kdpD are not essential (28-30) however it is plausible that combined mutations may be lethal. Alternatively the existence of an as yet unidentified essential c-di-AMP receptor is also entirely possible.

The c-di-AMP binding region in S. aureus KtrA and CpaA was narrowed down to the RCK_C domain (Figs. 2 and 4). This domain is present in a large number of bacterial and archaeal proteins and there is a good correlation between the distribution of the c-di-AMP cyclase domain DisA_N and the presence of RCK_C domains. Most bacteria and archaea that potentially synthesize c-di-AMP also contain one or more proteins with an RCK_C domain. This raises the possibility that c-di-AMP may contribute to the regulation of ion transport in a large number of bacteria and archaea. The number of RCK_C domains per organism usually exceeds the number of cyclases, perhaps suggesting that c-di-AMP regulates the function of multiple proteins, which is similar to what we found in S. aureus. However, the RCK_C domain is phylogenetically more widely distributed than the c-di-AMP cyclase domain and is also found in some eukaryotes such as green algae, in additional archaeal species and most notably in a large number of Gram-negative proteobacteria where the c-di-AMP cyclase domain is absent. We would predict that in those organisms other small molecules interact with this domain to regulate transport processes. The RCK_C domain is associated...
as a soluble domain with potassium transporters, or in some cases directly linked to ion antiporters, such as in CpaA. However, this domain is also associated with predicted amino acid antiporters, citrate transporters and voltage-gated channels. This suggests that c-di-AMP or other small molecules might regulate a range of different transport processes, which have not been previously associated with signaling networks.

Potassium is a major and essential intracellular ion and therefore bacteria have evolved several different types of uptake systems. The third c-di-AMP binding protein identified in this study was KdpD, which is a widely distributed membrane embedded sensor histidine kinase that in many bacteria controls, together with its response regulator KdpE, the expression of the second type of potassium uptake system. This ATP-dependent potassium uptake system has been best characterized in *E. coli* and consists of four membrane components KdpABC and the two-component system KdpDE, which is required for KdpABC expression at a very low potassium concentration when the other uptake systems are no longer sufficient to allow the cell to acquire the necessary amount of ion (31). However, a recent study on the *S. aureus* KdpDE system suggested that this two-component system has a different function in this organism (32). The *S. aureus* KdpDE two-component system, which still responds to the extracellular potassium concentration, was found to be no longer required for bacterial survival under low potassium conditions, but instead to control the expression of several well-characterized *S. aureus* virulence factors (32). However, additional work is needed to fully understand the function of this two-component system in *S. aureus* and other Gram-positive bacteria and based on this study its interplay with cellular c-di-AMP levels.

The least characterized c-di-AMP receptor protein identified in this study is the DUF970 domain-containing PII-like signal transduction protein, which belongs to the GlnB superfamily of proteins and was renamed PstA. PII-type proteins are one of the most widely distributed signal transduction proteins in nature that are present in bacteria as well as archaea and plants. DUF970 domain-containing PII-like proteins are not only present in *Staphylococcus* species but widely distributed among Firmicutes. Characterized proteins belonging to this GlnB superfamily are the cation tolerance protein CuaA (33) and the ATP phosphoribosyltransferase HisG, the first enzyme of the histidine pathway (34). However the best characterized proteins belonging to the GlnB superfamily are PII nitrogen regulatory proteins, which are key signal transduction protein that report on the nitrogen and carbon status of cells by sensing glutamine and 2-ketoglutarate levels (35). Since proteins belonging to this superfamily are known to bind diverse ligands and function by protein-protein interaction to control the activity of enzymes, transcription factor or transport proteins, we would assume that upon c-di-AMP binding or release the *S. aureus* PstA protein interacts with other cellular proteins. However these still need to be discovered.

This work demonstrates the feasibility of a DRaCALA-based ORFeome screen as a high-throughput platform for identifying c-di-AMP receptor proteins. While the DRaCALA ORFeome screen will identify receptors whose binding site does not require additional proteins, biochemical pull-down assays will only yield receptors that are expressed in the assayed growth conditions. Together the combination of biochemical pull-down assays, bioinformatic analysis and systems-wide screening of a whole genome protein expression library by DRaCALA provides a powerful synergistic approach for the systematic elucidation of protein-metabolite interaction networks (36). The discovery of the four different and widely distributed c-di-AMP receptor proteins allows future research to determine the molecular mechanisms underlying c-di-AMP dependent processes in prokaryotes.

**Methods**

**Bacterial strains and culture conditions.** *E. coli* strains were grown in LB or LB-M9 (37), *B. subtilis* strains in LB and S. aureus strains in TSB or chemically defined medium (CDM) at 37°C with aeration. CDM was prepared as referenced (38), with the following modifications: KHPO₄ was substituted with Na Phosphate buffer and KCl was added at concentrations stated in the text. In addition, Gly 50 mg/L; L-Ser 30 mg/L; L-Asp 90 mg/L; L-Lys 50 mg/L; L-Ala 60 mg/L; L-Trp 10 mg/L; L-Met 10 mg/L; L-His 20 mg/L; L-Ile 30 mg/L; L-Tyr 50 mg/L and thymine 20 mg/L were added. Information on strain construction is provided in the SI section. Strains and primers used are listed in Table S1 and S2 and the *S. aureus* (MRSA), Strain COL, Gateway Clone Set, Recombinant in Escherichia coli, Plates 1-25, NR-19277 were obtained through BEI Resources, NIAID, NIH.

**Affinity-pull down assay.** 20 ml of an *S. aureus* LAC* strain with an OΔO₂ operon was harvested and suspended in 1 ml 10 mM Tris HCl pH 7.5, 50 mM NaCl buffer containing EDTA-free complete protease inhibitor (Roche). Cells were mixed with 0.1 mm glass beads and lysed in a Fast-Prep machine twice for 45 sec. at setting 6 (MP Biomedicals). Samples were centrifuged for 5 min at 17,000 x g and subsequently for 1 h at 100,000 x g to obtain cytoplasmic...
protein extracts. 40 µl streptavidin dynabeads (Invitrogen) coupled with 2.4 µM biotinylated c-di-AMP (BioLog) were incubated with 1.2 mg cytoplasmic proteins in 1.5 ml 10% glycerol, 1 mM MgCl₂, 5 mM Tris pH 7.5, 230 mM NaCl, 0.5 mM DTT, 4 mM EDTA containing 50 µg/ml BSA for 30 min at room temperature. Samples were washed 4 times with the same buffer lacking BSA and resuspended in 1/10 of their original volume in 40 mM Tris pH 7.5, 100 mM NaCl, 10 mM MgCl₂. Binding buffer containing 2 mM PMFS, 20 µg/ml DNase and 0.5 mg/ml lysozyme. Cells were lysed by freeze thaw cycles. Lysates were directly used in binding assays or stored at −20°C.

Differential radial capillary action of ligand assay (DraCALA). The principle of the DraCALA is described in Roelofs et al (27). Briefly, E. coli whole cell lysates, 0.5 µM purified protein (for standard assays) or 12.5 µM protein extracts (for competition assays) in binding buffer were mixed with approximately 1 nM 32P-labeled c-di-AMP, synthesized as described in the supplementary information, or 5 nM 32P-labeled ATP and incubated at room temperature for 5 min. For the whole genome screen the 32P-labeled c-di-AMP was dispensed into lysate-containing 96 well plates using a Multiflo Microplate Dispenser (BioTek) and the mixture spotted onto nitrocellulose membranes using a 36 well pin tool (VLP Scientific). For competition assays, 100 or 400 µM cold nucleotides (ATP, GTP, cAMP, cGMP, NAD, NADH, NADP, NADPH, Sigma) c-di-AMP, c-di-GMP, BioLog were added to the initial mixture and 2.5 µl of reactions were spotted onto nitrocellulose membranes (American Hybrid-ECL GE Healthcare), air-dried and radioactivity signals detected as described above. The fraction of ligand bound and Ka values were calculated as previously described (27).

Acknowledgments. This research was supported by the European Research Council grant 260371 and the Wellcome Trust 100289 to A.G. and the EMBO short-fellowship 401-2011 to R.M.C.

4. Witte G, Hartung S, Buttrn K, Hopfner KP (2008) Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates. J Mol Biol 382(3):671-2.5 or 250 mM KCl. 100 µl of these suspensions were incubated in 96 well plates with 2 fold dilutions of Nigericin starting at 6.25 µM. Plates were incubated at 37°C with shaking for 24 h. MICs were determined as the minimum concentration at which growth was inhibited by >75% compared to growth without antimicrobial. Five independent experiments were performed and one representative graph is shown.

Bacterial Stress Testing. Overnight cultures of S. aureus strains in CDM containing 2.5 mM KCl were adjusted to 5 x 10³ bacteria/ml in gene-invariant production of c-di-AMP. Napgo F of Mot Mot 101(1):29-36.

Construction of the S. aureus ORfEm expression library, 2,343 E. coli strains containing pDONR221 vectors with S. aureus strain COL ORFs (BEI Resources, NASAID, NIH) were grown in 3.5 ml LB-M5 in 2 m 96-well deep dishes (Greiner) selecting for kanamycin resistance. The cultures were centrifuged and the plasmids extracted using 96-well MultiScreen® PLASMID plates (Millipore). The S. aureus gateway ORfEm library was shuttled from the pDONR221 plasmids into the protein overexpression destination vector pDEST17 using LR clonase enzyme II as per manufacturer's guidelines (Invitrogen). Subsequently, the destination plasmid library was introduced into E. coli strain T704 (NEB) selecting for carbencillin resistance.

Preparation of E. coli whole cell lysates. BL21(DE3) pET28b-containing strains or T704 pDEST17 containing library expression strains were grown in LB-M9 medium overnight at 30°C and subsequently induced for 6 h with 1 mM IPTG for protein induction. Bacteria were collected by centrifugation and suspended in 1/10 of their original volume in 40 mM Tris pH 7.5, 100 mM NaCl, 10 mM MgCl₂. Binding buffer containing 2 mM PMFS, 20 µg/ml DNase and 0.5 mg/ml lysozyme. Cells were lysed by freeze thaw cycles. Lysates were directly used in binding assays or stored at −20°C.

Differential radial capillary action of ligand assay (DraCALA). The principle of the DraCALA is described in Roelofs et al (27). Briefly, E. coli whole cell lysates, 0.5 µM purified protein (for standard assays) or 12.5 µM protein extracts (for competition assays) in binding buffer were mixed with approximately 1 nM 32P-labeled c-di-AMP, synthesized as described in the supplementary information, or 5 nM 32P-labeled ATP and incubated at room temperature for 5 min. For the whole genome screen the 32P-labeled c-di-AMP was dispensed into lysate-containing 96 well plates using a Multiflo Microplate Dispenser (BioTek) and the mixture spotted onto nitrocellulose membranes using a 36 well pin tool (VLP Scientific). For competition assays, 100 or 400 µM cold nucleotides (ATP, GTP, cAMP, cGMP, NAD, NADH, NADP, NADPH, Sigma) c-di-AMP, c-di-GMP, BioLog were added to the initial mixture and 2.5 µl of reactions were spotted onto nitrocellulose membranes (American Hybrid-ECL GE Healthcare), air-dried and radioactivity signals detected as described above. The fraction of ligand bound and Ka values were calculated as previously described (27).

Acknowledgments. This research was supported by the European Research Council grant 260371 and the Wellcome Trust 100289 to A.G. and the EMBO short-fellowship 401-2011 to R.M.C.