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Helicase Motif III in SecA is essential for coupling preprotein binding to translocation ATPase

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The SecA ATPase is a protein translocase motor and a superfamily 2 (SF2) RNA helicase. The ATPase catalytic core ('DEAD motor') contains the seven conserved SF2 motifs. Here, we demonstrate that Motif III is essential for SecA-mediated protein translocation and viability. SecA Motif III mutants can bind ligands (nucleotide, the SecYEG translocase 'channel', signal and mature preprotein domains), can catalyse basal and SecYEG-stimulated ATP hydrolysis and can be activated for catalysis. However, Motif III mutation specifically blocks the preprotein-stimulated 'translocation ATPase' at a step of the reaction pathway that lies downstream of ligand binding. A functional Motif III is required for optimal ligand-driven conformational changes and kinetic parameters that underlie optimal preprotein-modulated nucleotide cycling at the SecA DEAD motor. We propose that helicase Motif III couples preprotein binding to the SecA translocation ATPase and that catalytic activation of SF2 enzymes through Motif-III-mediated action is essential for both polypeptide and nucleic-acid substrates.

Keywords: SecA; ATPase; DNA/RNA helicase; protein translocation; signal peptide

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INTRODUCTION

The bacterial preprotein translocase comprises a SecA ATPase bound to a SecYEG membrane 'pore' (Hartl *et al*, 1990; Veenendaal *et al*, 2004; Vrontou & Economou, 2004).

SecA, a unique bifunctional enzyme, is a protein translocase motor as well as an RNA helicase (Park *et al*, 1997). SecA has the DEAD (Asp-Glu-Ala-Asp) motor ATPase core (Fig 1A,B) of superfamily 2 (SF2) helicases (Koonin & Gorbalenya, 1992; Sianidis *et al*, 2001; Caruthers & McKay, 2002; Hunt *et al*, 2002; Sharma *et al*, 2003), in which two subdomains (domains 1 and 2) form a mononucleotide cleft (Caruthers & McKay, 2002). In SecA, these are nucleotide-binding domain (NBD; amino acids (aa) 1–220 and 378–420) and intramolecular regulator of ATPase (IRA2; aa 421–610; Sianidis *et al*, 2001; Baud *et al*, 2002; Hunt *et al*, 2002; Sharma *et al*, 2003). Seven conserved SF2 motifs (Fig 1B) line the ATP cleft (see Discussion) and a subset of these (e.g., Motifs I, II, V and VI) are involved in ATP binding and catalysis (Mitchell & Oliver, 1993; Sianidis *et al*, 2001). Two unique 'enzymatic specificity' appendages distinguish SecA from other helicases: one that 'sprouts out' of NBD (substrate specificity domain (SSD), aa 221–377; Baud *et al*, 2002) and another that is fused carboxy terminally to IRA2 and binds to both NBD and IRA2 (C-domain, aa 611–901; Fig 1A).

Secretory preproteins bind to SecA (Hartl *et al*, 1990), probably at SSD (Kimura *et al*, 1991; Baud *et al*, 2002), and activate its ATPase activity (Lill *et al*, 1990), allowing processive preprotein translocation (Vrontou & Economou, 2004). ATP catalysis and preprotein binding are repressed in cytoplasmic SecA by the C-domain IRA1 switch (Fig 1; Karamanou *et al*, 1999) and IRA1 derepression is required to activate nucleotide and preprotein binding and ATP catalysis (Vrontou *et al*, 2004). The mechanism by which preproteins activate ATP hydrolysis at a distance is unclear.

Motif III of SF2 helicases contains the conserved tripeptide (Thr/Ser-Gly/Ala-Thr/Ser). The function of this tripeptide is poorly understood (Caruthers & McKay, 2002), but it has been implicated in coupling RNA binding to helicase activity (Pause & Sonenberg, 1992; Linder *et al*, 2000). Motif III of SecA, however, is known to be required for its RNA helicase activity (Schmidt *et al*, 2001). We now examine the possible additional role of helicase Motif III (Fig 1A–C) in protein translocation. We find that SecA Motif III couples preprotein-induced conformational effects to ATPase stimulation and is a novel essential region of protein translocase.

RESULTS

Motif III is essential for protein translocation

To investigate the role of the SecA helicase Motif III (^{391}TGT^{393}), we performed alanine-scanning mutagenesis (Fig 1C). In addition, Thr393Asn was constructed. The ability of mutant genes to rescue a *secA*^{ts} strain (BL21.19; Mitchell & Oliver, 1993), when carried

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on a plasmid, was examined at 42 °C (Fig 1D). *secA*^{T391A} and *secA*^{G392A} complemented the *ts* strain, although less efficiently than *secA,* whereas neither *secA*^{T393A} nor *secA*^{T393N} did.

For biochemical analysis, oligohistidine-tagged SecA and N68 (i.e. SecA aa 1–610; Karamanou *et al*, 1999) derivatives were generated. All proteins were purified to homogeneity (except SecA^{T393A}, which was rapidly degraded during purification and was not studied further). Motif III mutants were dimeric, folded and extensively α-helical, as was wild-type (WT) SecA (data not shown).

We then examined the ability of SecA Motif III mutants to support protein translocation *in vitro* (Fig 1E). SecA^{T391A} (lane 5) and SecA^{G392A} (lane 6) catalysed proOmpA translocation into SecYEG proteoliposomes, albeit not as efficiently as SecA (lane 3). In contrast, SecA^{T393N} (lane 7) could not do so at any concentration tested (supplementary Fig S1 online).

These data demonstrate that Motif III is important for SecA-dependent protein translocation. Thr 393 in particular is essential. To probe Motif III function further, we focused on SecA^{T393N}.

SecA^{T393N} binds nucleotide

Motif III lies within the nucleotide cleft (Fig 1A; Hunt *et al*, 2002; Sharma *et al*, 2003). To examine whether it contributes to nucleotide binding, we compared the equilibrium dissociation constants (K_D) of SecA^{T393N} and N68^{T393N} for ADP with those of WT SecA and its N68 derivative. Mutant and WT proteins showed similar affinities (supplementary Table S1 online), suggesting that Motif III is not an essential determinant of DEAD motor nucleotide binding.

SecA^{T393N} binds SecYEG

Binding of SecA to SecYEG is essential for protein translocation (Hartl *et al*, 1990). To examine whether Motif III is critical for such binding, we compared the K_D of SecA for SecYEG with that of SecA^{T393N} (supplementary Table SII online). At 4 °C, SecA^{T393N} binds to SecYEG present on the inner membrane vesicles (IMVs) with high but marginally reduced affinity compared with SecA. At 37 °C, both proteins bind to SecYEG with indistinguishable affinities. These data demonstrate that SecA^{T393N} binds proficiently to SecYEG.

SecA^{T393N} binds preprotein

To examine whether Motif III mutation affects SecA–preprotein interaction, we used 3K7L (synthetic signal peptide; Miller *et al*, 1998; Baud *et al*, 2002) and a model substrate CH5EE (the mature domain of an M13 coat derivative that binds SecA, outcompetes proOmpA binding and requires SecA for its membrane insertion; Roos *et al*, 2001). We compared the ability of SecA and SecA^{T393N} to bind immobilized 3K7L or CH5EE using an optical biosensor and found that both bind to either 3K7L (Fig 2A) or CH5EE (Fig 2B) to similar extents, whereas the unrelated maltose-binding protein does not.

Binding of 3K7L or CH5EE to N68 suppresses its ATPase activity. N68^{T393N} ATPase was suppressed by either signal peptide (Fig 2C) or CH5EE (Fig 2D) as efficiently as that of N68.

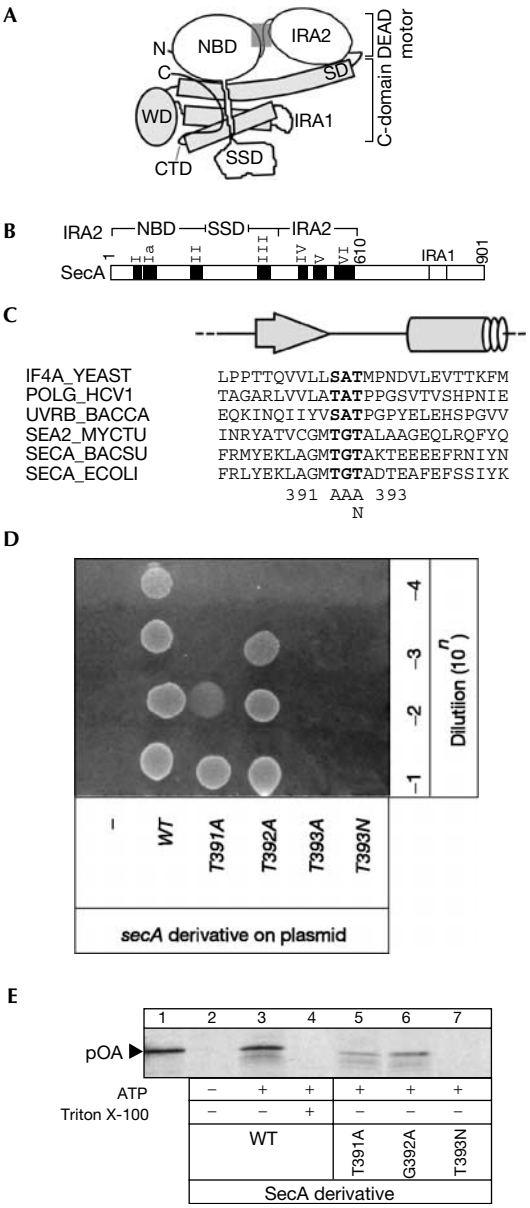

Fig 1 | Helicase Motif III is essential for protein translocation. (**A**) Model of a SecA protomer. CTD, C-terminal domain; IRA, intramolecular regulator of ATPase; NBD, nucleotide-binding domain; SD, scaffold domain; SSD, substrate specificity domain; WD, wing domain. The grey square represents Motif III. (**B**) SF2 motifs (Roman numerals; Sianidis *et al*, 2001; Caruthers & McKay, 2002) are indicated on a SecA linear map. (**C**) Comparison of SF2 Motif III sequences from RNA helicases eIF4A (yeast) and NS3 (hepatitis C virus), DNA repair protein UvrB (*Bacillus caldotenax*) and SecA (*Escherichia coli, Mycobacterium tuberculosis* and *Bacillus subtilis*), below a secondary structure schematic (cylinder, α-helix; arrow, β-strand). Conserved residues are in bold. Generated substitutions are shown below. (**D**) Complementation of the *secA*^{ts} strain BL21.19 by pET5 or pET5 carrying cloned *secA*s. Indicated culture dilutions (grown at 30 °C) were spotted on LB/ampicillin plates and incubated (42 °C). (**E**) proOmpA (pOA) translocation in SecYEG proteoliposomes catalysed by WT SecA or SecA Motif III mutants (40 μg/ml) as described (Karamanou *et al*, 1999). Lane 1, 30% of undigested input [^{35}S]proOmpA; lanes 2–7, treated with proteinase K. Translocated proOmpA became protease-accessible when Triton X-100 (1% v/v) was added before proteolysis (lane 4).

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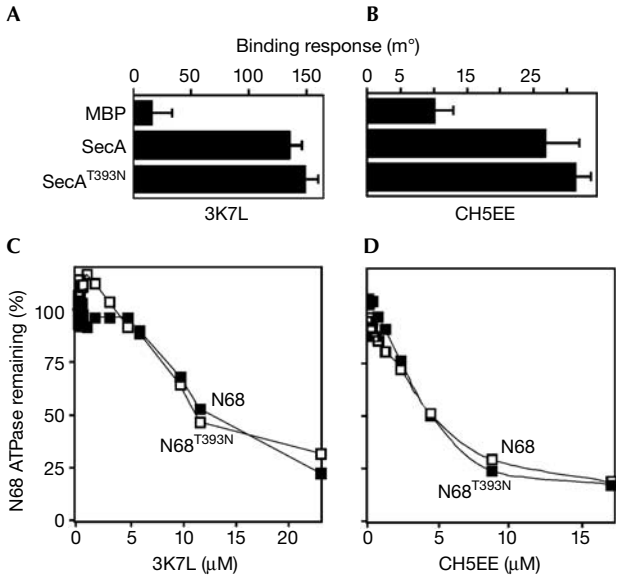

Fig 2 | Preprotein binding to SecA^{T393N}. (**A**,**B**) SecA and SecA^{T393N} binding to a signal peptide (3K7L (**A**); Baud *et al*, 2002) or a mature domain (CH5EE (**B**); Papanikou *et al*, in preparation) optical biosensor. MBP, maltose-binding protein, used as a control. (**C**,**D**) N68 or N68^{T393N} (buffer B, 1 mM ATP) was supplemented with increasing amounts of 3K7L (**C**) or CH5EE (**D**). Basal ATPase was determined (30 min; 37 °C; Lill *et al*, 1990). ATPase activities are expressed as a percentage of the activity of N68 in the absence of peptides.

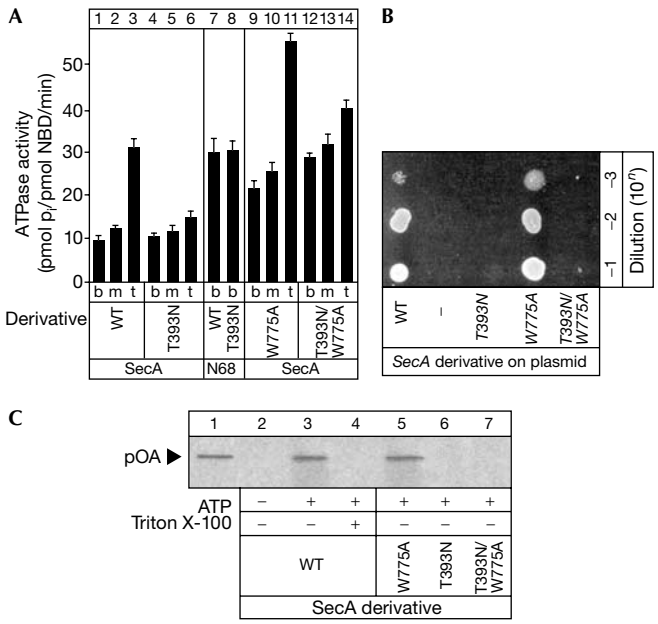

Fig 3 | Poor stimulation of SecA^{T393N} ATPase by preprotein. (**A**) Basal (b), membrane (m) and translocation (t) ATPase activities of SecA or N68 derivatives (Lill *et al*, 1990). (**B**) Genetic complementation of the *secA^{ts}* strain BL21.19 (as in Fig 1D). (**C**) *In vitro* proOmpA (pOA) translocation in SecYEG proteoliposomes catalysed by SecA or derivatives (as in Fig 1E).

Taken together, both physical and functional assays suggested that the T393N mutation did not affect preprotein binding to SecA.

SecA^{T393N} ATPase is barely stimulated by preprotein

To probe further the role of Motif III, the basal, membrane (i.e. SecYEG-stimulated) and translocation (i.e. SecYEG- and preprotein-stimulated) ATPase activities (Lill *et al*, 1990) of SecA^{T393N} were determined (Fig 3A). Basal and membrane ATPases of SecA^{T393N} were similar to those of SecA (compare lanes 4 and 5 with lanes 1 and 2, respectively). However, the translocation ATPase of SecA^{T393N} (lane 6) was only poorly stimulated (27% more than its membrane ATPase; lane 5) in contrast to that of SecA (~190%; compare lane 3 with 2). This inability does not reflect an inherent defect of the mutant DEAD motor to perform high-level ATP hydrolysis as the ATPase of N68^{T393N} was as high as that of N68 (compare lane 8 with 7).

IRA1 derepression is a necessary step that activates translocase ligand binding and ATP hydrolysis (Karamanou *et al*, 1999; Vrontou *et al*, 2004). To investigate whether the defect of SecA^{T393N} (Fig 2; supplementary Tables SI and SII online) relates to this mechanism, we artificially bypassed it using the IRA1 mutation W775A (Vrontou *et al*, 2004). We found that W775A does not compromise viability (Fig 3B) or protein translocation (Fig 3C, lane 5), but leads to high SecA basal ATPase (Fig 3A, lane 9) and preprotein binding (Baud *et al*, 2002; Vrontou *et al*, 2004). W775A was also introduced in the SecA^{T393N} mutant. The basal and membrane ATPase activities of SecA$^{T393N/W775A}$ and SecA^{W775A} are similar (Fig 3A, compare lanes 12 and 13 with lanes 9 and 10). However, unlike SecA^{W775A}, Preprotein binding to SecA^{AT343N} fails to stimulate significantly its membrane ATPase on preprotein addition (compare lane 14 with 11; ~133 versus ~29% stimulation), to complement the *secA^{ts}* strain (Fig 3B) or to support *in vitro* protein translocation (Fig 3C, lane 7).

Our data demonstrate that SecA^{T393N} is defective in acquiring preprotein-stimulated ATPase. This defect is unrelated to IRA1 derepression.

Altered ligand-induced SecA^{T393N} conformations

To explain our results, we considered the hypothesis that although SecA^{T393N} binds preprotein and nucleotide it may be unable to acquire the appropriate conformation required for ATP catalysis. To test this, SecA and SecA^{T393N} were trypsinized under identical conditions (Fig 4) in the absence of ligands (lane 1), in the presence of CH5EE (lane 2) or ADP (lane 3), or in the presence of both ligands (lane 4). The digests were then immunostained with antibodies raised against either the IRA2 or SSD subdomains (Baud *et al*, 2002; Vrontou *et al*, 2004). Two characteristic tryptic fragments were monitored: p16 (aa 420–586) detected by anti-IRA2 antibodies (Fig 4A) and p12 (aa 220–329) detected by anti-SSD antibodies (Fig 4B).

In the absence of any ligand, trypsin cleavage in SecA generates p16 (Fig 4A, lane 1); addition of the model substrate CH5EE does not alter this pattern (lane 2). When ADP is added, alone (lane 3) or together with CH5EE (lane 4), p16 is no longer generated. In SecA^{T393N}, generation of p16 persists even when ADP is added, either alone (lane 3) or together with CH5EE (lane 4).

Conversely, generation of p12 from SecA is only seen following CH5EE addition, alone (Fig 4B, lane 2) or together with ADP (lane 4). In SecA^{T393N}, only negligible amounts of p12 are generated

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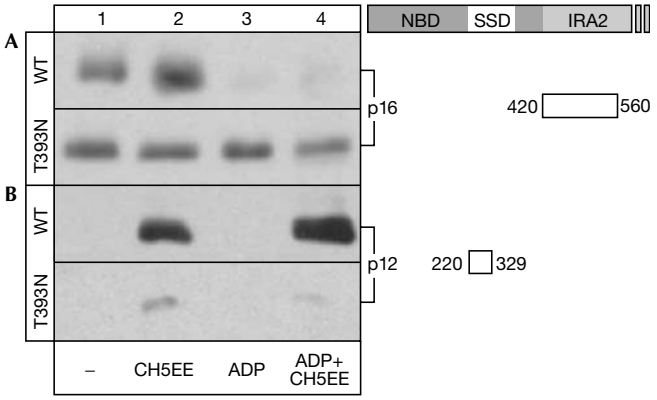

Fig 4 | Ligand-induced conformation of SecA and SecA^{T393N} (20 μg) were trypsinized (16 μg/ml; buffer B; 2.5 min; 4 °C) in the absence of ligands or in the presence of CH5EE (19.5 μM) or ADP (10 μM), or both. Trypsin was inactivated by Pefabloc (9 mM). Polypeptides were separated by SDS–polyacrylamide gel electrophoresis and immunostained by anti-IRA2 (p16) or anti-SSD (p12) domain-specific antibodies (Baud *et al*, 2002).

under the same conditions (compare SecA and SecA^{T393N} in lanes 2 and 4).

Our data indicate that translocation ligands drive distinct conformational states in SecA. SecA^{T393N} binds these ligands but fails to acquire the appropriate ligand-driven conformations that SecA does.

Altered preprotein-induced ADP cycling in SecA^{T393N}

The inability to acquire the appropriate ligand-induced conformations might compromise SecA nucleotide turnover capability. We tested this by directly comparing nucleotide cycling kinetic rates in the presence or absence of CH5EE in SecA and SecA^{T393N} (supplementary Table SIII online). In the absence of CH5EE, the association (k_{on}) and dissociation (k_{off}) rates for MANT-ADP are indistinguishable between SecA and SecA^{T393N}. Binding of CH5EE does not affect k_{on} of MANT-ADP to SecA but slows down k_{on} in SecA^{T393N}. Moreover, whereas binding of CH5EE seems to retard k_{off} of MANT-ADP from SecA, the same event enhances k_{off} in SecA^{T393N}.

Our results indicate that nucleotide cycling kinetics is under preprotein-binding control in SecA. This mechanism is altered in SecA^{T393N}.

DISCUSSION

To gain further insight into SecA-mediated protein translocation, we examined the role of helicase Motif III. Mutagenesis showed Motif III to be an important SecA region and in particular Thr 393 to be essential for viability (Fig 1D) and protein translocation (Fig 1E). Despite these severe effects, SecA^{T393N} is either not affected or only modestly affected with respect to ligand binding, whether to nucleotide (supplementary Table SI online), signal peptide (Fig 2A,C), the mature domain of a model preprotein (Fig 2B,D) or SecYEG (supplementary Table SII online). Moreover, SecA^{T393N} retains the basal and membrane ATPase properties of SecA (Fig 3A, compare lane 1 with 4 and lane 2 with 5) and N68^{T393N} is as efficient as N68 in ATP hydrolysis (Fig 3A, lanes 7 and 8). These results suggest that Motif III is neither involved in ligand binding nor in basic ATP catalysis.

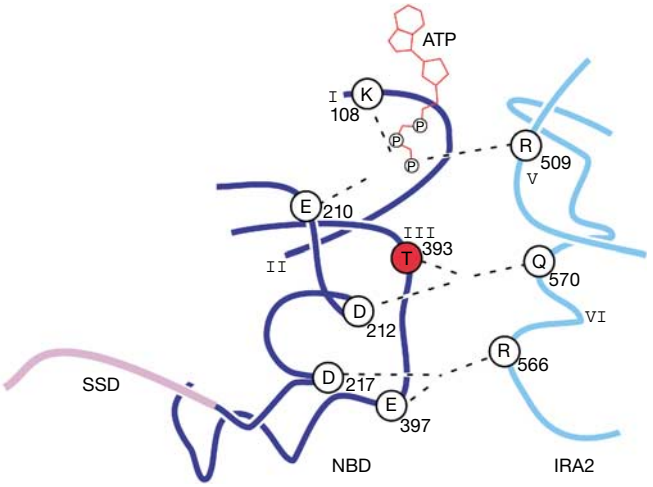

Fig 5 | Interactions between Motif III and other SF2 motifs in SecA (Hunt *et al*, 2002; Sharma *et al*, 2003). See text for details.

SecA^{T393N} is specifically compromised in preprotein-stimulated ATPase (Fig 3A, compare lane 6 with 3). This defect persists even when IRA1 is artificially derepressed (Fig 3A, compare lane 14 with 11). SecA^{T393N} cannot acquire the ligand-driven conformations that the SecA DEAD motor does (Fig 4). As a functional repercussion, preprotein-regulated nucleotide cycling in SecA^{T393N} is altered (supplementary Table SIII online). These data suggest that a general feature of preproteins may be to modulate DEAD motor conformations and that these become coupled through Motif III to nucleotide exchange/catalysis. An interesting analogy exists in the eIF4A SF2 RNA helicase where Motif III mutations affect helicase activity without affecting ATP catalysis or RNA binding (Pause & Sonenberg, 1992; Linder *et al*, 2000).

Motif III, located strategically at the interface of NBD and IRA2, intertwines (Fig 5) with the catalytic Motif II (Asp 212) and IRA2 (Gln 570) (Hunt *et al*, 2002; Sharma *et al*, 2003). These interactions may affect (i) ATP γ-phosphate hydrolysis through proximal residues (e.g. Glu 210/Arg 509) and (ii) NBD–IRA2 communication either directly (Gln 570) or through Asp 217/Glu 397 that pair with the crucial Arg 566 (Sianidis *et al*, 2001). NBD–IRA2 communication lies at the heart of SecA mechanics because it activates DEAD motor ATPase and controls the rate-limiting ADP release step (Sianidis *et al*, 2001). Hence, Motif III may link nucleotide binding/hydrolysis to NBD–IRA2 association/dissociation. It is tempting to speculate that preproteins gain control of the catalytic cycle through the physical links of SSD with Motifs II and III (Fig 5).

Motif III/Motif II interactions are seen in other SF2 (Cho *et al*, 1998; Theis *et al*, 1999; Story *et al*, 2001) and SF1 (Velankar *et al*, 1999) helicases. Nevertheless, the primary sequence and length of Motif III and of its interacting 'domain 2' residues are not conserved between SF2 and SF1 helicases (Caruthers & McKay, 2002). SF1 helicase Motif III is elongated and interacts directly with nucleic-acid substrates (Korolev *et al*, 1997). These observations suggest that Motif III allows optimal DEAD motor response to individual substrates. Remarkably, SecA Motif III is required for both RNA helicase (Park *et al*, 1997; Schmidt *et al*, 2001) and protein translocase (Fig 1E) activities. This extraordinary

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example of enzymatic adaptation shows that the two seemingly unrelated chemistries may be catalysed by similar DEAD motor mechanisms.

METHODS

Strains and DNA manipulation. Strains, growth and DNA manipulation were as described (Mitchell & Oliver, 1993; Karamanou *et al*, 1999). Site-directed mutations were generated using the 'megaprimer' PCR method (Ke & Madison, 1997). To generate 'megaprimers', we used the forward primer X26 (5′-GCGGGATCCGCGCGCTGCTG-3′) and the reverse primers X114 (5′P-GAAAGCTTCGGTATCAGCAGCACCGGTCATCCCCG-3′) for T393A mutation, X115 (5′P-CTTCGGTATCAGCAGTAGCGGTCATCCCCGCCAG-3′) for G392A mutation, X117 (5′P-GTATCAGCAGTACCAGCCATCCCCGCCAGTTTTTC-3′) for T391A mutation, X138 (5′P-GAAAGCTTCGGTATCAGCATTACCGGTCATCCCCG-3′) for T393N mutation. The 'megaprimers' were then used in a second PCR reaction together with X130 (3′-CCGTCCATGCAGTGCTACGCCCTAGGCAGCGC-5′). Products from the second PCR were digested with *Bam*HI/*Bgl*I, gel purified and inserted in the corresponding sites of *secA* in pIMBB7 (HisSecA), giving rise to pIMBB124 ($SecA^{T391A}$), pIMBB128 ($SecA^{G392A}$), pIMBB129 ($SecA^{T393A}$) and pIMBB239 ($SecA^{T393N}$). Cloning and purification of CH5EE (Roos *et al*, 2001) will be described elsewhere (Papanikou *et al*, in preparation).

Nucleotide exchange kinetics. Association rate (k_{on}) was determined at 37 °C using 0.2 μM MANT-ADP (Molecular Probes) and 0.2 μM SecA or $SecA^{T393N}$. CH5EE was added at 1 μM. Once equilibrium was attained, the dissociation rate (k_{off}) of bound MANT-ADP was determined on addition of 1 mM ATP. Data were collected (0–600 s; excitation, 356 nm; emission, 450 nm; slits, 2.5/5) using a Cary Eclipse (Varian, Australia) fluorimeter (Vrontou *et al*, 2004).

Miscellaneous. Chemicals were from Sigma (USA), DNA enzymes from Minotech (Greece), oligonucleotides from MWG (Germany), [^{35}S]methionine (1,000 Ci/mmol) and chromatography materials from Amersham (Sweden), and Ni^{2+}-NTA resin from Qiagen. Protein purification, biochemical assays and antibodies were as described previously (Lill *et al*, 1990; Sianidis *et al*, 2001; Baud *et al*, 2002; Vrontou *et al*, 2004). Binding and kinetic data were analysed using Prism (GraphPad, USA) or Origin (Microcal, USA).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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