RESEARCH COMMUNICATION

14-3-3 proteins are required for the inhibition of Ras by exoenzyme S

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14-3-3 proteins play a regulatory role and participate in both signal transduction and checkpoint control pathways. 14-3-3 proteins bind phosphoserine ligands, such as Raf-1 kinase and Bad, by recognizing the phosphorylated consensus motif, Arg-Ser-Xaa-pSer-Xaa-Pro (where 'Xaa' represents 'any residue', and 'pSer' is 'phosphoserine'). However, 14-3-3 proteins must bind unphosphorylated ligands, such as glycoprotein Ib α and *Pseudomonas aeruginosa* exoenzyme S (ExoS), since it has been suggested that specific residues of 14-3-3 proteins are required for activation of ExoS. Furthermore, an unphosphorylated peptide derived from a phage display library inhibited the binding of both ExoS and Raf-1 to 14-3-3, and bound within the same

conserved amphipathic groove on the surface of 14-3-3 as the Raf-derived phosphopeptide (pS-Raf-259). In the present study we identify the interaction site on ExoS for 14-3-3, and show that ExoS and 14-3-3 do indeed interact *in vivo*. In addition, we show that this interaction is critical for the ADP-ribosylation of Ras by ExoS, both *in vitro* and *in vivo*. Loss of the 14-3-3 binding site on ExoS results in an ExoS molecule that is unable to efficiently inactivate Ras, and displays reduced killing activity.

Key words: ADP-ribosylation, cystic fibrosis, ExoS, *Pseudomonas aeruginosa*.

INTRODUCTION

14-3-3 proteins are a group of highly conserved helical, intracellular dimeric molecules expressed in plants, invertebrates and higher eukaryotes with several ascribed functions, which include involvement in neurotransmitter biosynthesis, cell cycle control, and development and survival in yeast and mammalian fibroblasts [1–5]. 14-3-3 proteins are suggested to mediate in signal transduction by binding to phosphoserine-containing proteins, such as tyrosine and tryptophan hydroxylase, Raf-1, cdc25, kinase suppressor of Ras-1, protein kinase C and the Bcl-2 family member Bad, with a defined consensus binding motif of Arg-Ser-Xaa-pSer-Xaa-Pro (where 'Xaa' represents 'any residue', and 'pSer' is 'phosphoserine'), or Arg-Xaa-(Tyr/Phe)-Xaa-pSer-Xaa-Pro [6,7].

Furthermore, Petosa et al. [8] have shown that an unphosphorylated peptide, 'R18', binds within the same amphipathic groove of 14-3-3. Thus 14-3-3 proteins are also capable of binding unphosphorylated molecules, such as glycoprotein Ib α and exoenzyme S (ExoS) *in vitro* [9,10]. ExoS is a bifunctional toxin encoded by the *Pseudomonas aeruginosa* pathogen [11]. It has a C-terminal ADP-ribosylation activity (residues 232–453), which blocks receptor-stimulated Ras activation *in vivo* and has been reported to contain an N-terminal (residues 1–234) Rho GTPase-activating protein (GAP) activity *in vitro* [12,13].

Here we identify the interaction site on ExoS for 14-3-3, and show that ExoS and 14-3-3 do indeed interact within the intact cell. Moreover, we show that this interaction is critical for the ADP-ribosylation of Ras by ExoS both *in vitro* and *in vivo*. Finally, we show that loss of the 14-3-3 binding site results in an ExoS molecule that is unable to efficiently inactivate Ras, and displays reduced killing activity.

EXPERIMENTAL

Cell cultures, cell lysis and [³⁵S]methionine labelling

HeLa and K562 cells were grown in RPMI 1640 medium

supplemented with 10 % (v/v) fetal-bovine serum and 100 units/ml penicillin. Cells were washed in cold PBS, and were lysed on ice in lysis buffer [1 % (v/v) Triton X-100/100 mM NaCl/50 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 mM EGTA/1 mM PMSF] supplemented with protease inhibitors (10 μ g/ml aprotinin, pepstatin and leupeptin). Lysates were cleared by centrifugation at 15000g for 10 min at 4 °C, and precleared with 5 μ g of glutathione-agarose (Amersham Pharmacia Biotech, Uppsala, Sweden)-coupled glutathione S-transferase (GST)-fusion protein. Equal amounts of lysate were incubated with primary antibody or GST-fusion proteins for 1 h, and with Protein G-agarose or glutathione-agarose for a further 30 min. After four washes in lysis buffer, samples were boiled in SDS/PAGE sample buffer. HeLa cell extracts were labelled by overnight incubation with 0.25 μ Ci of [³⁵S]methionine (SJ1015; Amersham Pharmacia Biotech)/10-cm Petri dish in RPMI 1640-methionine, 1 % (v/v) fetal-bovine serum, 1 mg/ml BSA, 20 mM Hepes, pH 7.5. Precipitations were performed as described above.

Western-blot analysis and antibodies

Anti-(14-3-3 β) was purchased from Santa Cruz (New York, NY, U.S.A.); monoclonal Ras (R02120) was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Monoclonal 9E10 antibody was kindly given by Dr J. Downward (Imperial Cancer Research Fund, London, U.K.). Immunoblotting was performed according to the manufacturer's instructions using secondary antibodies conjugated to horseradish peroxidase sheep anti-mouse antibody (ECL Plus; Amersham Pharmacia Biotech).

Plasmids, GST-protein expression and transfections

All ExoS derivatives used were constructed from pTS103 and pTS106 [11] as templates for PCR. Primers were designed to introduce flanking 5'-BamHI and 3'-EcoRI restriction sites (underlined in the subsequent sequences). Amplified cDNA was inserted into a pGEX-2TK vector (Amersham Pharmacia Bio-

Abbreviations used: ExoS, exoenzyme S; GAP, GTPase-activating protein; GST, glutathione S-transferase.

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tech) at *Bam*HI–*Eco*RI restriction sites to produce GST-fusion proteins. Primers used were as follows: for GST–ExoS(88–453) and GST–ExoS(E381A), 5′-CCA GTG GAT CCG CGC AGC CTG CGG T-3′ (P1) and 5′-TAC GAC GAA TTC GGC CAG ATC AAG GC-3′ (P2); for GST–ExoS(88–375), P1 and 5′-GTT GAA TTC CCC GCT GAC ATC GAT TCC-3′; for GST–ExoS(88–312), P1 and 5′-TTC GAA TTC TCC GCT CTT CTC GAA GGC-3′; for GST–ExoS(300–453), 5′-GCC GGA TCC AAG AGC GGA CAG GCT GAA C-3′ and P2; for GST–ExoS(366–453), 5′-GGA GGA TCC GTC AGC GGG ATA TCG AAC-3′ (P3) and P2; for GST–ExoS(387–453), 5′-CAA AGG ATC CGA CAT GCG CGT GCT GCT G-3′ and P2; for GST–ExoS(400–453), 5′-GAT GGA TCC GGA GTG ACC CGC CGG GTT C-3′ and P2; for GST–ExoS(366–426), P3 and 5′-CAG GAA TTC AGC ATC GAG CAG TCC CTG-3′ (P4); for ExoS(88–426), P1 and P4; for GST–ExoS(222–453), 5′-GTT GGG ATC CGC GGT AGA GAG CGA GGT C-3′ (P5) and P2; and for GST–ExoS(222–426), P5 and P4. Subsequently, all constructs were sequenced (UBI sequencing kit; Lake Placid, NY, U.S.A.). pGEX-2T-14-3-3-ζ and pRSET-Ha-Ras were kindly given by Dr Alastair Aitken, (Edinburgh, Scotland, U.K.) and Dr Julian Downward respectively, and expressed as described previously [14,15]. For mammalian expression, ExoS fragments –(264–1359), –(264–1278) and –(1098–1359) were excised from the respective GST–ExoS fusion vectors (*Bam*HI–*Eco*RI) and inserted into the pCS3-MT vector [16,17] at *Bgl*II–*Eco*RI restriction sites. Transfection of HeLa cells was performed using LipofectAMINE[®] (Gibco BRL, Gaithersburg, MD, U.S.A.) according to the manufacturer's instructions and K562 cells were transfected as described in [18].

ExoS activation assay

The reaction mixtures contained (in a final vol. of 20 μl): 0.2 M sodium acetate, pH 6.0, 10 μM purified Ha-Ras, 0.9 nM 14-3-3 protein, 70 nM GST–ExoS or its mutant proteins, and 1.25 mM NAD⁺ (Sigma–Aldrich, Stockholm, Sweden). After 60 min at 37 °C, reactions were stopped by adding 20 μl of SDS/PAGE sample buffer and boiling for 5 min. The samples were then subjected to SDS/PAGE and immunoblotting.

RESULTS AND DISCUSSION

During our previous examination of the modification *in vivo* of Ras elicited by the toxin ExoS in HeLa and NIH3T3 cells, we were unable to detect any direct interaction between Ras proteins and ExoS (results not shown; also see [12]). To address the question of which of the cellular proteins ExoS is capable of interacting with, we employed a GST-fusion protein 'pull-down' approach using pre-labelled [³⁵S]methionine HeLa cell lysates. This resulted in the detection of two predominant and specific bands that approximated to the molecular mass of the Ras GTPase, which specifically bound to GST–ExoS(88–453), but not to GST alone, GST–ExoS(88–312) or GST–YopE (Figure 1A, upper panel, lane 3 compared against lanes 2, 4 and 5). We employed the *Yersinia* toxin, YopE, in our experiments since it has recently been suggested that an N-terminal region, corresponding to amino acids 1–234 of both ExoS and YopE, which are 54% homologous, function as GAPs for the Rho GTPase family [11,13]. Immunoblotting of the above [³⁵S]methionine-labelled blot and GST-fusion pull-downs from unlabelled HeLa cells revealed that the detected bands were not Ras or Rho proteins (results not shown). Further investigation identified 14-3-3 proteins as being those that bound to ExoS (Figure 1A, lower panel).

Figure 1 GST–ExoS interacts with endogenous 14-3-3 proteins

HeLa cells were harvested, and lysates were subjected to 'pull-down' analysis with 5 μg of various GST-fusion proteins. Samples were separated on an SDS/12.5% polyacrylamide gel. (A) Upper panel: samples from pull-downs employing different GST-fusion proteins from [³⁵S]methionine-labelled HeLa cell lysates. Lane 1, unlabelled control lysate; lane 2, GST alone; lane 3, GST–ExoS(88–453), containing the YopE homology and the ADP-ribosylation domains of ExoS; lane 4, GST–ExoS(88–312) containing the domain of ExoS with homology to the toxin YopE; lane 5, GST–YopE. Lower panel: samples from pull downs (as above) probed with anti-(14-3-3) antibodies. (B) Upper panel: HeLa cell lysates were subjected to affinity precipitation with a GST–ExoS-deletion series, with the numbering of the lanes corresponding to the schematic representations of the constructs illustrated in the lower part of (B). 14-3-3 proteins were detected by immunoblotting with monoclonal anti-(14-3-3) antibodies. Lower panel: diagram detailing the different GST-fusion protein constructs of ExoS used in the present study. Important domains of ExoS are indicated as follows: amino acids 232–453, designated by the box labelled as 'ADP-ribosylation dom.', signifies the catalytic ADP-ribosyltransferase domain; the filled-in box (amino acids 1–232) describes the region of limited similarity shared between ExoS and the *Yersinia* virulence factor, YopE [11].

It has been proposed that the eukaryotic host factor that activates ExoS of *P. aeruginosa* *in vitro* is a member of the 14-3-3 protein family [19,20]. Furthermore, a non-phosphorylated peptide, R18, from a phage-display library can inhibit the interaction between 14-3-3 and Raf-1 or ExoS, and also blocks the activation of ExoS by 14-3-3 *in vitro* [8,10]. The finding that a GST–ExoS fusion protein interacts with 14-3-3 proteins from cell lysate raises the question of whether direct interaction

Figure 2 14-3-3 and ExoS interact *in vivo*

Left panel: Western-blot analysis of whole K562 extracts transiently transfected with Myc-tagged ExoS derivatives. Lane 1, Myc-ExoS(88–453); lane 2, Myc-ExoS(88–426); lane 3, Myc-ExoS(366–453). Right panel: immunoprecipitation with agarose-conjugated Myc antibodies after transient transfection of Myc-ExoS derivatives in K562 cells, followed by Western-blot analysis, as described in the Experimental section. Lane 1, Myc-ExoS(88–453), lane 2, Myc-ExoS(88–426); lane 3, Myc-ExoS(366–453). Lower panel: membranes were stripped and reprobed with anti-(14-3-3) antibody, as indicated. Molecular-mass markers (in kDa) are shown on the right.

between 14-3-3 and ExoS is necessary for the ExoS activity and its subsequent modification of Ras. In order to assess this question, we constructed a GST-ExoS-deletion series (Figure 1B; lower panel), which was used in a GST-fusion protein pull-down experiment. HeLa cells were lysed, and lysates were pre-cleared with GST proteins to remove proteins with non-specific binding, followed by precipitation with the GST-ExoS fusion proteins, as indicated (Figure 1B; upper panel). Samples were run on an SDS/polyacrylamide gel, followed by immunoblotting with anti-(14-3-3) antibodies. All GST-ExoS derivatives lacking any portion of the C-terminal part of ExoS failed to interact with and precipitate 14-3-3 proteins, i.e. GST-ExoS(88–375), GST-ExoS(88–312), GST-ExoS(400–426) and the GST construct lacking only the C-terminal 27 amino acids, GST-ExoS(88–426). However, all constructs, including the most C-terminal part of ExoS, were able to efficiently precipitate 14-3-3 proteins, i.e. GST-ExoS(88–453), GST-ExoS(300–453), GST-ExoS(366–453), GST-ExoS(387–453) and GST-ExoS(400–453). Functional analysis *in vitro* of ExoS has associated the ADP-ribosylation activity with residue position 381, and mutation of this site, ExoS(Glu³⁸¹ → Ala) [or ExoS(E381A)] has been shown to decrease the catalytic activity 2000-fold [11,21]. This inactive ExoS mutant does not abrogate the activation of Ras, extracellular signal-regulated kinase or protein kinase B/Akt upon stimulation with epidermal growth factor [12]. However, GST-ExoS(E381A) is still able to interact efficiently with 14-3-3 proteins, and therefore ExoS ADP-ribosylation activity is not required for the 14-3-3-ExoS interaction.

To investigate the interaction between 14-3-3 and the C-terminal part of ExoS *in vivo*, we transfected K562 cells with Myc-tagged ExoS derivatives, immunoprecipitated using anti-Myc antibodies followed by immunoblotting for endogenous

Table 1 Cellular viability was measured after transfection with 10 μ g of pCS3-MT, pCS3[ExoS(88–453)]-MT or pCS3[ExoS(88–426)]-MT

K562 cells (1×10^6) were transfected with each construct, and 24 and 48 h after transfection, cells were stained with 0.4% Trypan Blue and examined under visible light for the ability to exclude Trypan Blue. Data are from at least four independent transient transfections and quantification of three random micrographs for each transfection of non-Trypan-Blue-stained cells was determined as the percentage compared with the control construct, pCS3-MT.

DNA construct	Time (h) after transfection	
	24	48
pCS3-MT	100	100
pCS3[ExoS(88–453)]-MT	75 $\pm$ 6	44 $\pm$ 3
pCS3[ExoS(88–426)]-MT	96 $\pm$ 5	95 $\pm$ 4

14-3-3 (Figure 2). Both ExoS(88–453) and ExoS(366–453) were able to precipitate 14-3-3 proteins from the cell lysate. However, the ExoS(88–426) derivative, lacking the last 27 amino acids, did not interact with and immunoprecipitate 14-3-3 proteins in this assay (Figure 2, right panel: compare lane 5 with lanes 4 and 6). Furthermore, immunoprecipitation of endogenous Ras from ExoS(88–453)-transfected cells resulted in a slower migrating form of Ras on SDS/PAGE, thus confirming Ras as a substrate for ExoS ADP-ribosylation activity *in vivo*. This was not observed when Ras was precipitated from cells transfected with empty vector or ExoS(88–426) (results not shown). Thus we have characterized the interaction between 14-3-3 and the non-phosphorylated ExoS further, and we show that the 14-3-3 binding site is located in the last 27 amino acids of the ExoS protein.

During the course of these experiments, we observed that ExoS(88–453) has severely toxic effects on cells when compared with the other ExoS constructs used. Noticeably fewer cells survived transfection using the full-length ExoS construct, resulting in significantly less Myc-tagged ExoS(88–453) protein after harvesting compared with cells transfected with Myc-tagged ExoS(88–426) and Myc-tagged ExoS(400–453) (Figure 2; left panel). This was investigated further using cell counting and Trypan Blue exclusion assays. Data from at least four independent transient transfection experiments with ExoS(88–453) revealed that only 44% of the cells were viable after 48 h; this was not observed when cells were transiently transfected with ExoS(88–426) or with empty vector (pCS3) (Table 1). Frithz-Lindsten et al. [11] showed, using the *Y. pseudotuberculosis* type III secretion/translocation system, that 45% of the cells infected with ExoS after 5 h were non-viable, and that prolonged infection leads to lethality and detachment of cells from the Petri dish surface. Our results suggest that transfected ExoS(88–426), without the 14-3-3 interaction domain, shows no cytotoxic effect in K562 cells, and is indistinguishable from the transiently transfected empty vector. However, ExoS(88–453), containing the complete catalytic domain (including the 14-3-3 interaction domain), is enzymically active and promotes cell killing.

To analyse whether the C-terminal 27 amino acids of ExoS are important for both the ADP-ribosylation activity and modification of Ras by ExoS, different GST-ExoS protein constructs were incubated with *in vitro* transcription/translated [³⁵S]-methionine-incorporated Ras proteins. The GST-ExoS(88–426) fusion protein was defective in its capacity to modify Ras *in vitro* (Figure 3A, lane 3). However, GST-ExoS(88–453) is capable of modifying Ras efficiently within 1 h (Figure 3A; compare lane 2 with lanes 1 and 3). All modification assays of Ras *in vitro* by

Figure 3 ExoS modification of Ras requires the 14-3-3 binding site

(A) Ha-Ras was labelled with [35 S]methionine in an *in vitro* transcription/translation system (Promega), and proteins were separated on an SDS/12% polyacrylamide gel, and analysed by autoradiography. Lane 1, wild-type Ha-Ras alone; lane 2, Ha-Ras incubated with GST-ExoS(88-453); lane 3, Ha-Ras incubated with GST-ExoS(88-426). (B) Bacterially expressed and purified His-tagged Ha-Ras proteins were mixed with various GST-ExoS constructs, which had (+) or had not (–) previously been tumbled in HeLa extracts. Samples were incubated for 60 min at 37 °C, and, thereafter, were separated using SDS/12.5% PAGE. Upper panel: GST alone (lane 1), GST-ExoS(88-453) (lanes 2 and 3), GST-ExoS(88-426) (lanes 4 and 5), GST-ExoS(222-453) (lanes 6 and 7), GST-ExoS(222-426) (lanes 8 and 9). Samples were analysed by immunoblotting with anti-Ras monoclonal antibodies. Lower panel: the above membrane was stripped and analysed by immunoblotting with anti-(14-3-3) polyclonal antibodies. (C) Ha-Ras was incubated with GST (lane 1), GST-ExoS(88-453) (lanes 2 and 3), GST-ExoS(E381A) (lanes 5 and 6) or GST-ExoS(88-426) fusion proteins (lanes 7 and 8), together with purified (+) or non-purified (–) 14-3-3 for 1 h at 37 °C. Samples were separated by SDS/PAGE, followed by immunoblotting with anti-Ras monoclonal antibody.

ExoS derivatives were performed for 1 h, although the effects are clearly observed to be completed after 5 min with ExoS constructs containing both the catalytic and the 14-3-3 binding domain (results not shown). Another approach was to mix bacterially purified Ha-Ras proteins, NAD⁺ and purified GST-ExoS deriva-

tives, which were tumbled in HeLa extracts for 30 min, prior to separation on SDS/PAGE. Ras proteins mixed with the 'dipped' ExoS(88-453) samples showed a slower mobility on SDS/PAGE (Figure 3B, lane 3) compared with Ras proteins mixed with dipped ExoS(88-426) (Figure 3B, lane 5), indicating that ExoS is able to extract a binding protein important for activity from HeLa lysate. GST and GST-ExoS(88-453), which was not dipped in cell lysate, showed no ability to modify Ras (Figure 3B, lanes 1 and 2). To further confirm the importance of the C-terminus of ExoS, we constructed GST-ExoS(222-453) and GST-ExoS(222-426), both lacking the N-terminal YopE homology domain of ExoS, and these were employed in our 'fishing' analysis, with similar results as those found with the wild-type sequence (Figure 3B, upper panel, lanes 6–9). Further investigation by immunoblotting shows that the GST-ExoS derivatives with a 14-3-3 binding domain successfully interacted with, and precipitated, 14-3-3 proteins from the cell lysate (Figure 3B, lower panel, lanes 3 and 7). In every case, GST-ExoS proteins, which were unable to bind and pull out 14-3-3, were also unable to modify Ras.

As a final test for the ExoS activity with or without the C-terminal 27 amino acids, we developed an *in vitro* assay with purified ExoS, Ha-Ras and 14-3-3 proteins, together with NAD (Figure 3C). We show that incubation of Ha-Ras, 14-3-3 and GST alone does not change the mobility of Ras proteins (Figure 3C, lane 1). However, when Ha-Ras and 14-3-3 were mixed with ExoS(88-453), Ras modification was observed (Figure 3B, lane 3). As suspected, when ExoS(E381A) or ExoS(88-426) were mixed together with Ha-Ras and 14-3-3 proteins, no change was observed in the mobility of Ras (Figure 3C, lanes 5 and 7). Thus using purified components *in vitro* we are able to show that binding of 14-3-3 by ExoS is required for the efficient modification, and thus inhibition, of Ras.

In summary, our results show that the bacterial toxin ExoS is unequivocally an *in vivo* interaction partner with 14-3-3 proteins upon infection with *P. aeruginosa*. In addition, we have demonstrated here that the 14-3-3 binding epitope is within the last 27 non-phosphorylated amino acids of ExoS. This interaction is in disagreement with earlier predictions that the interaction occurs in the vicinity of amino acid 245 on ExoS [10]. C-terminally deleted ExoS is significantly less cytotoxic than ExoS with an intact 14-3-3 binding domain. We show that the ability of ExoS and the C-terminal deletion mutant to modify Ras correlates with the viability of cells infected with these ExoS proteins *in vivo*. Although 14-3-3 proteins have been convincingly demonstrated to associate with signalling molecules, and now also with active bacterial toxins, the functional role of these proteins in these signalling pathways remains complex, and is not fully understood. However, it may be speculated that 14-3-3 proteins are not only used as cofactors for ExoS activities, but can also be 'hijacked' and used as guidance molecules for toxins, such as ExoS, in order to find their target substrates upon translocation from the bacteria to the host cell.

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