Exoenzyme S shows selective ADP-ribosylation and GTPase-activating protein (GAP) activities towards small GTPases *in vivo*

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Intracellular targeting of the *Pseudomonas aeruginosa* toxins exoenzyme S (ExoS) and exoenzyme T (ExoT) initially results in disruption of the actin microfilament structure of eukaryotic cells. ExoS and ExoT are bifunctional cytotoxins, with N-terminal GTPase-activating protein (GAP) and C-terminal ADP-ribosyltransferase activities. We show that ExoS can modify multiple GTPases of the Ras superfamily *in vivo*. In contrast, ExoT shows no ADP-ribosylation activity towards any of the GTPases tested *in vivo*. We further examined ExoS targets *in vivo* and observed that ExoS modulates the activity of several of these small GTP-binding proteins, such as Ras, Rap1, Rap2, Ral, Rac1, RhoA and Cdc42. We suggest that ExoS is the major ADP-ribosyltransferase protein modulating small GTPase function encoded by *P. aeruginosa*. Furthermore, we show that the GAP activity of ExoS abrogates the activation of RhoA, Cdc42 and Rap1.

Key words: bacterial toxin, cytotoxicity, cystic fibrosis, GTP-binding protein, *Pseudomonas aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that causes infections in immunocompromised individuals, such as patients with burn wounds or leukaemia, and can cause chronic infections in cystic fibrosis patients [1–4]. One virulence strategy used by the opportunistic pathogen *P. aeruginosa* is to target toxic proteins into eukaryotic cells by a type III secretion mechanism, a delivery mechanism utilized by several Gram-negative bacteria and plant pathogens [5–9]. Two *P. aeruginosa* toxins that are secreted and translocated into eukaryotic cells are exoenzyme S (ExoS) and exoenzyme T (ExoT) [10]. These proteins are highly homologous and both have been shown to possess ADP-ribosylation activities *in vitro* [8,11]. Previous studies show that ExoT, like ExoS, alters the cell morphology of Chinese hamster ovary cells [6,12,13] and inhibits uptake by corneal epithelial cells [14], but the exact role of ExoT during infection is unknown.

ExoS is able to ADP-ribosylate Ras and several related proteins *in vitro* [15,16], while no ADP-ribosylation target for ExoT has been identified. ExoS is a bifunctional toxin; in addition to its C-terminal ADP-ribosylation activity *in vivo* [6,17–19], ExoS has an N-terminal Rho GTPase-activating protein (GAP) activity *in vitro* [20]. In addition, a eukaryotic factor activating ExoS, or FAS factor, is required to activate ExoS in cells [21]. FAS is a member of the 14-3-3 protein family [22], and this interaction between 14-3-3 and ExoS is critical for Ras ADP-ribosylation by ExoS [21–23]. ExoT also possesses a C-terminal ADP-ribosyltransferase domain *in vitro* [10,24] and an N-terminal Rho-GAP domain, which shows efficient GAP activity towards Rho GTPases *in vitro* [12,25,26].

To investigate further the mechanism of ExoS and ExoT action/toxicity, we have focused on the identification of *in vivo* targets for the ExoS and ExoT ADP-ribosylation activities. Ras and related proteins belong to a superfamily of small GTP-binding proteins, which can be divided into five subfamilies: Ras, Rho, ADP-ribosylation factors (ARFs), Rab and Ran. The Ras subfamily is involved in cell proliferation, differentiation, as well as progression through the G_1 phase and cell survival [27,28]. The Rho family with its three major subfamilies, Rho, Rac and Cdc42, is involved in the control and reorganization of the cytoskeleton, as well as regulation of gene transcription [29,30]. The ARF GTPase family is an important player in several vesicular trafficking pathways [31]. The Rab family, which contains at least 30 members, is involved in the secretory and endocytic pathways. Rab GTPases facilitate the formation of v-SNARE–t-SNARE complexes (where SNARE is soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor, v is vesicle and t is target), which are integral components of vesicle trafficking [31]. Finally, the Ran subfamily plays a role in nuclear trafficking [32].

In this study we show that ExoS is a promiscuous ADP-ribosyltransferase. *In vitro*, ExoS is able to modify all GTPases tested. However, *in vivo* infection of eukaryotic cells with ExoS does not result in ADP-ribosylation of RhoA, RhoB, RhoD, Rab4, Rab27, TC21, ARF3 or Ran. In contrast, the related ExoT shows no detectable *in vivo* ADP-ribosylating activity towards any of the GTPases tested. Further evidence for ExoS being the major ADP-ribosylation activity towards the small GTPases *in vivo* comes from analysis of cells infected with different *P. aeruginosa* strains. We show that deletion of the gene encoding exoS results in an inability of *P. aeruginosa* to modify any small GTPases *in vivo*. We further show that the bifunctional ExoS protein not only modifies, but also modulates, the activation state of several proteins, such as Ras, Ral, Rap1, Rap2,

Abbreviations used: ARF, ADP-ribosylation factor; BD, binding domain; ExoS, exoenzyme S; ExoT, exoenzyme T; FCS, fetal calf serum; GAP, GTPase-activating protein; GDS, GDP/GTP dissociation stimulator; GST, glutathione S-transferase; IBMX, 3-isobutyl-1-methylxanthine; LPA, lysophosphatidic acid; PAK, p21-activated kinase; PDGF, platelet-derived growth factor; WASP, Wiskott–Aldrich syndrome protein.

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Rac1, RhoA and Cdc42 *in vivo*. In conclusion, we suggest that the function of ExoS *in vivo* is to perturb activation of not just one, but multiple small GTP-binding proteins and their effector signal-transduction pathways.

MATERIALS AND METHODS

Cell cultures and bacterial strains

HeLa cells were grown as described previously [12]. Bacterial strains used in this report, *P. aeruginosa* strains 388 (wild-type) [11], 388ΔexoS::Tc [33], 388exoT::Tc [10], *Yersinia pseudotuberculosis* expressing ExoS [YPIII(pIB251, pTS103)], ExoS(E381A) [YPIII(pIB251, pTS106)], ExoS(Δ98-232) [YPIII(pIB251, pTS103; Δ98-232)], ExoT (YPIII 251, pCS100) and for mock infection, YPIII(pIB251), have been described previously [12,19]. Bacterial cultivation and infection procedures were carried out as described previously [12].

Plasmids and antibodies

pcDNA3-MT-Rab4, pSG5-HA-RalA, pCDNA3-MT-Rap1, pGEM-MT-Rab5a, pRK5-MT-R-Ras and pcDNA3-MT-RhoA, B and D were kindly given by Dr M. Cormont, Dr H. Gille, Dr A. Hall and Dr H. Mellor. pMT-HA-Rap1, pMT-HA-Rap2 and pMT-HA-PDZGEF were kind gift of Dr B. Burgering. Anti-Ras and Ras-related antibodies were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Polyclonal ExoS antibodies were produced by Agrisera (Umeå, Sweden) [6]. Antibodies towards TC21, RhoA, Cdc42, R-Ras, K-Ras and N-Ras were purchased from Santa Cruz Biotechnology. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), lysophosphatidic acid (LPA), ionomycin and bradykinin were purchased from Sigma. Epidermal growth factor was from UBI (Lake Placid, NY, U.S.A.) and platelet-derived growth factor (PDGF) was from R&D Systems. Glutathione–Sepharose 4B was obtained from Amersham Biosciences.

Transfection, cell lysis and immunoprecipitation

HeLa cells were transfected in 100 mm plates using the LIPOFECTAMINE™ kit (Gibco BRL) according to the manufacturer's instructions. Cells were washed twice in ice-cold PBS and lysed on ice in lysis buffer [1 % Triton X-100, 100 mM NaCl, 50 mM Tris/HCl, pH 7.5, and 1 mM EDTA, supplemented with protease inhibitors (10 μg/ml aprotinin, pepstatin and leupeptin)]. Lysates were cleared by centrifugation at 15000 ***g*** for 10 min at 4 °C. Equal amounts of cell lysates were incubated with 5 μg of anti-Myc antibodies cross-linked to Protein G beads as described [34]. After four washes in lysis buffer, samples were boiled in SDS sample buffer followed by immunoblotting. pMT-HA-Rap1, pMT-HA-Rap2 and pMT-HA-PDZGEF were transfected as above but harvested as described in [35].

In vitro modification of Ras superfamily

GTPases were [^{35}S]methionine-labelled (Amersham Biosciences catalogue no. PB10282) by *in vitro* transcription/translation in a coupled reticulocyte lysate system according to the manufacturer's instructions (TNT®T7 coupled reticulocyte lysate system; Promega). Each translation mix (2 μl) was then incubated with reaction buffer and 2 μl of secreted ExoS, as described previously [6,19]. Samples were analysed by SDS/PAGE (12.5 % gels) followed by autoradiography.

Actin staining

The cells were grown on collagen I-coated 8-well culture slides (0.5×10^5 cells/well; Becton-Dickinson). At time points after infection, 1 h and overnight, the cells were fixed with 2 % paraformaldehyde for 5 min, washed twice with PBS and permeabilized with 0.15 % saponin for 10 min. The actin microfilaments were stained with FITC-conjugated phalloidin for 30 min at 37 °C. Coverslips were mounted in a mounting medium containing CitiFluor (City University, London, U.K.) as an antifading agent. Disruption of the actin microfilaments was analysed using fluorescence microscopy.

In vivo Ral, Rap1/2, Rac, Rho, Cdc42 and Ras activation assays

GTPase fusion proteins were purified and employed as described previously: GST-Raf1-Ras-BD [36], GST-RalGDS-Rap1/2-BD [37,38], GST-PAK-Rac-BD and GST-WASP-Cdc42-BD [39], GST-Rhotekin-Rho-BD [40] and GST-RalBP1-Ral-BD [41] (where GST is glutathione S-transferase, RalGDS is Ral GDP/GTP dissociation stimulator, PAK is p21-activated kinase, WASP is Wiskott–Aldrich syndrome protein and BD is binding domain). Cells were stimulated and infected as indicated in the Figures. Equal volumes of lysates were incubated with GST-fusion protein beads at 4 °C for 1 h, washed and bound GTPase was separated on SDS/PAGE, followed by immunoblotting. Whole-cell lysates were also analysed for the presence of GTPase, as indicated in the Figures. Depending on cell conditions and different batches of GST-GTPase-BD, the GTPase-bound protein accounts for 1–5 % of total GTPase. Each series of infection and subsequent GST-fusion pull-down assay was carried out at least three times for each endogenous GTPase.

RESULTS

ExoS is a promiscuous ADP-ribosylation enzyme *in vitro*

It is well established that Ras is a target for ExoS ADP-ribosylation activity [15,17,42–48]. Since there is a high similarity among the family members of the small GTPases, we investigated whether small GTP-binding proteins other than Ras could also be modified by ExoS. To achieve this, we first tested ExoS for its *in vitro* ability to ADP-ribosylate a number of small GTPases: Ha-Ras, RalA, RhoA, RhoB, RhoD, Cdc42, Rac1, Rab5, Rab4 and Rap1. GTPases were ^{35}S-labelled *in vitro* and tested for ADP-ribosylation modification activity using ExoS (Figure 1). We and others have earlier reported that the slower-migrating Ras protein is a consequence of ADP-ribosylation by ExoS [6,15,17,19]. Interestingly, Ras, Rap1, Rap2 and Ral contain a conserved arginine residue at amino acid 41, which has been identified as the target for ADP-ribosylation in Ras [17,44]. While many members of the Ras superfamily do not contain an arginine at amino acid 41, they do contain arginine residues in flanking sequence regions, which are potential sites for ADP-ribosylation. In our experiments all tested GTP-binding proteins show a slower mobility after incubation with ExoS (Figure 1). Thus ExoS is able to modify all GTPases tested *in vitro* [15].

ExoS ADP-ribosylates multiple GTPases *in vivo*

We have previously shown that ExoS ADP-ribosylates Ras, using the *Y. pseudotuberculosis* type III secretion system as a tool to deliver ExoS proteins into cells [19]. The advantage of using the *Yersinia* system is the opportunity to study the effects of ExoS and ExoT in the absence of other toxins. HeLa cells were infected with *Y. pseudotuberculosis* expressing either ExoS or ExoT as described previously [6,12]. In addition, we also infected HeLa

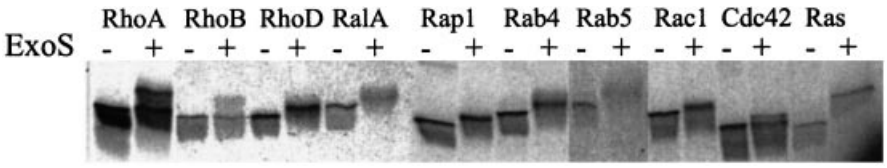

Figure 1 ExoS ADP-ribosylation activity *in vitro*

Various small GTP-binding proteins, as indicated, were [^{35}S]methionine-labelled by *in vitro* transcription/translation and mixed with bacterially secreted ExoS, as described in the Materials and methods section, and thereafter separated by SDS/PAGE (12.5% gels) and detected by autoradiography.

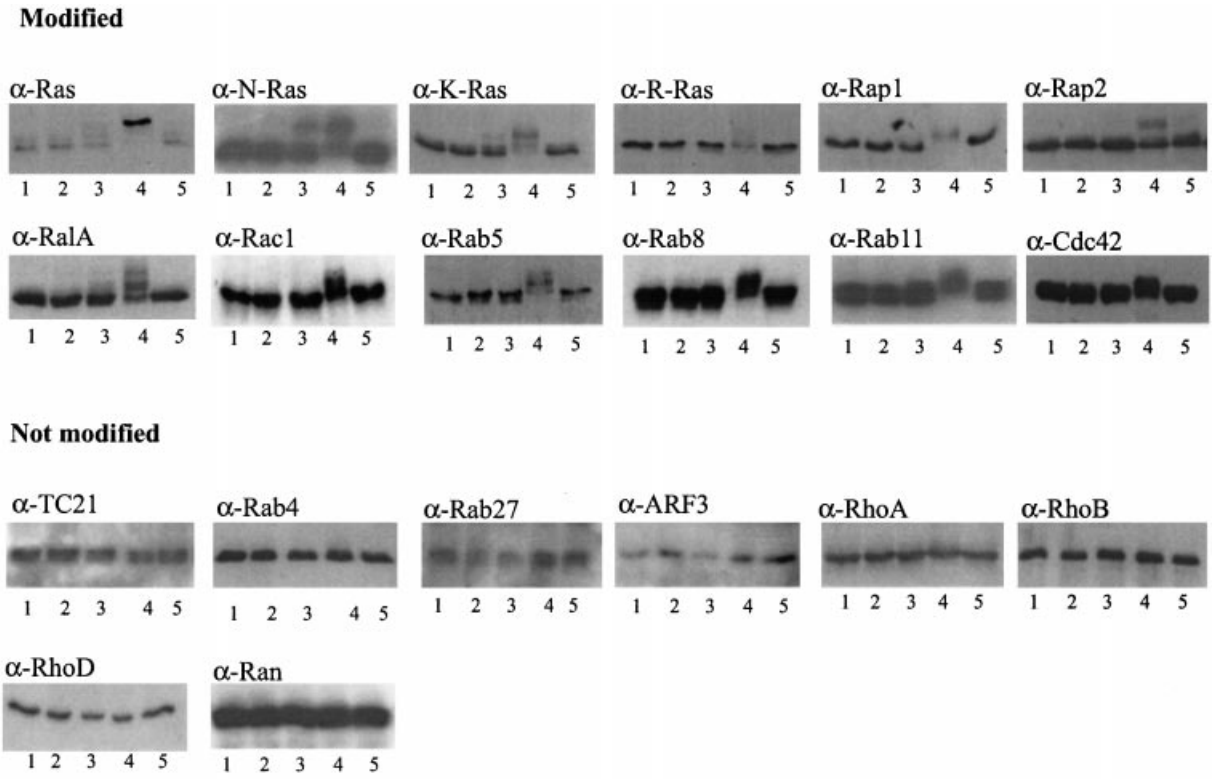

Figure 2 ADP-ribosylation activities of ExoS and ExoT *in vivo*

HeLa cells were infected for 80 min with *Yersinia* expressing different variants of ExoS and ExoT. Cells were lysed and samples were separated by SDS/PAGE (12.5% gels). Western-blot analysis was performed on immunoblotted filters towards GTPases as indicated. Lanes 1, uninfected; lanes 2, mock infection; lanes 3, ExoS(E381A); lanes 4, ExoS; lanes 5, ExoT.

cells with bacteria expressing ExoS(E381A), a mutation (Glu-381→Ala) that decreases ADP-ribosylation activity 2000-fold [6,18]. After infection, HeLa cell lysates were analysed by immunoblotting against a variety of endogenous small GTPases (Figure 2). We also examined HeLa cells transiently transfected with Myc-tagged RhoD, which were subsequently immunoblotted with anti-Myc antibodies (Figure 2). The effect of ExoS on the different Ras-related proteins was surprising; we observed that H-Ras, N-Ras, K-Ras, R-Ras, Rap1, Rap2, Rab5, Rab8, Rab11, RalA, Cdc42 and Rac1 are ADP-ribosylated by wild-type ExoS *in vivo* (Figure 2, lanes 4). We were unable to observe modification of TC21, Ran, RhoA, RhoB, RhoD, ARF3, Rab4 or Rab27 (Figure 2), although slight modification of RhoA by ExoS can be seen after a 120 min infection. However, at this time point, HeLa cells detach from the Petri dish and initiate death pathways (results not shown). Thus the relevance of RhoA modification at this time point is questionable. Furthermore, transiently transfected Myc-tagged RhoD was not modified either (Figure 2). No significant modification of the small G-proteins was observed using the ExoS(E381A) mutant (Figure 2, lanes 3). In contrast with ExoS, ExoT resulted in no detectable *in vivo* ADP-ribosylation of any of the small GTPases examined (Figure 2, lanes 5).

ExoS is the major *P. aeruginosa* ADP-ribosylation toxin targeting host cell GTPases *in vivo*

The current literature regarding ExoS and ExoT function describes both as ADP-ribosylation enzymes [10,24]. We wished to address the issue of whether ExoS or ExoT is responsible for GTPase modification by the *P. aeruginosa* host bacterium itself. This is an important question, since the relevance of ADP-ribosylation of ExoS and ExoT by *P. aeruginosa* during infection is unclear. In order to investigate this, we utilized the following

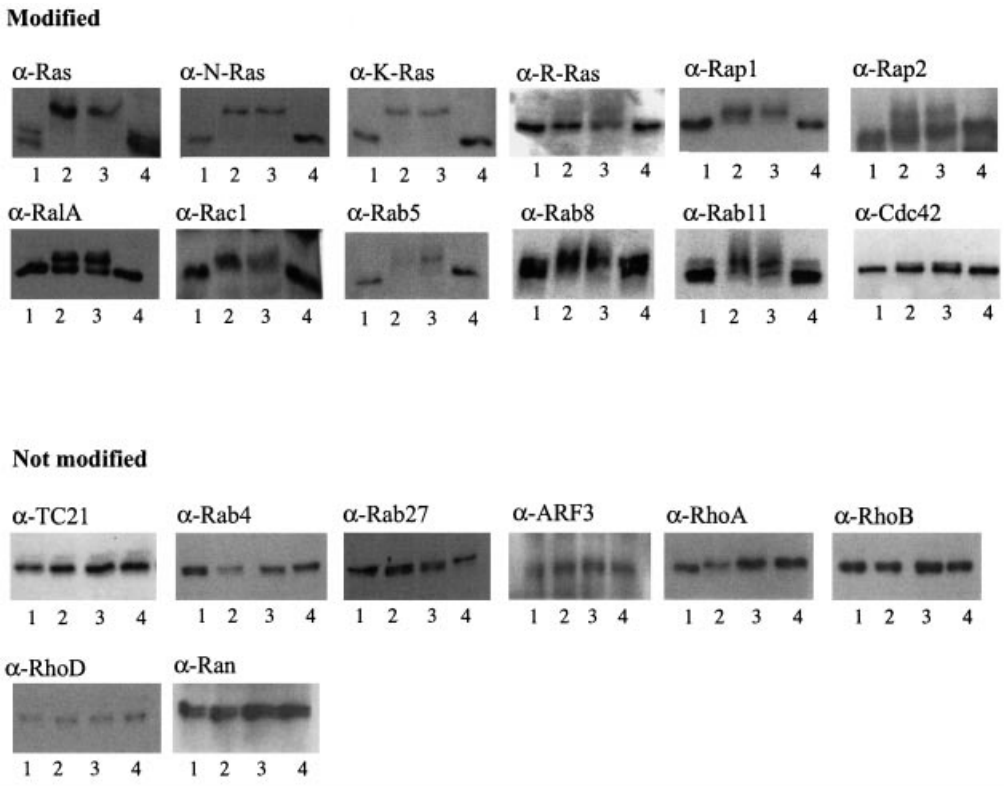

Figure 3 ExoS is the major *Pseudomonas* ADP-ribosylation activity towards small GTPases *in vivo*

HeLa cells were infected for 2.5 h with *P. aeruginosa* strains expressing both ExoS and ExoT (388), ExoS (388exoT::Tc) or ExoT (388ΔexoS::Tc). Cells were lysed and samples were separated by SDS/PAGE (12.5% gels). Western-blot analysis was performed on immunoblotted filters as indicated in the Figure. Lanes 1, uninfected; lanes 2, 388 wild-type; lanes 3, 388exoT::Tc; lanes 4, 388ΔexoS::Tc.

P. aeruginosa strains: (i) 388 wild-type, expressing both ExoS and ExoT [11], (ii) 388ΔExoT, expressing ExoS but not ExoT [10], and (iii) 388ΔExoS, which expresses ExoT but not ExoS [33]. This panel of *P. aeruginosa* strains was used to infect HeLa cells and to study the *in vivo* effect of ExoS and ExoT on multiple members of the Ras superfamily. In order to assess GTPases modification *in vivo*, HeLa cells were infected for 2.5 h. Lysates were separated on SDS/PAGE and immunoblotted with anti-Ras and Ras-related antibodies as before (Figure 3).

Our results were identical with those of our previous infections (Figure 2), where the type III secretion system of *Yersinia* delivered ExoS and ExoT. In the case of infection by *P. aeruginosa* 388 (wild type) and 388ΔExoT strains, both of which express and secrete ExoS, small GTPases were modified in a promiscuous manner. We observed that while TC21, ARF3, Ran, RhoA, RhoB, RhoD, Rab4 and Rab27 were not modified by ExoS *in vivo*, all other Ras and Ras-related proteins tested were modified by the *P. aeruginosa* strains 388 and 388ΔExoT (Figure 3, lanes 2 and 3). This implies that ExoT is not required *in vivo* for ADP-ribosylation of the small GTPases. If ExoT is not required for this modification *in vivo*, one would then predict that a strain mutated for *exoS*, but still carrying a functional *exoT* gene, would not be capable of modifying Ras-family proteins *in vivo*. This is in fact the case, since analysis of HeLa cells infected by the *P. aeruginosa* 388ΔExoS strain, which carries and expresses ExoT but not ExoS, is completely unable to modify any of the small GTPases analysed *in vivo* (Figure 3, lanes 4). Since loss of ExoS expression completely abrogates the ability of *P. aeruginosa* to target and modify the small GTPases tested *in vivo* we conclude that ExoS is the major ADP-ribosylating activity directed against host small GTPases encoded by *P. aeruginosa*.

ExoS ADP-ribosylation activity *in vivo*

We wished to address whether the different ExoS variants used above resulted in changes in the morphology of the infected cells. To achieve this, we used ExoS, ExoS(E381A) and ExoS(Δ98-232), the last of which is a mutant where the GAP domain has been deleted [19]. The function of this panel of ExoS proteins was assayed *in vivo* by infection of HeLa cells, allowing us to ask whether the ADP-ribosylation or the GAP activities of ExoS have functional relevance (Figure 4). To assess changes in cytotoxicity and morphology *in vivo*, HeLa cells were infected for 1 h with ExoS and ExoS mutant variants. After 1 h we observed morphological changes in the infected cells. All cells infected with ExoS, ExoS(E381A) and ExoS(Δ98-232) show a more rounded-up morphology compared with uninfected cells (Figure 4). These results clearly point to an *in vivo* function for both the GAP domain and the ADP-ribosylation domain of ExoS on morphology and actin reorganization.

To investigate further the important role for survival, morphology and actin reorganization of the GAP and the ADP-ribosylation domains of ExoS upon infection, we performed a second experiment in which HeLa cells were infected for 1 h with ExoS and derivatives of ExoS and subsequently washed free of

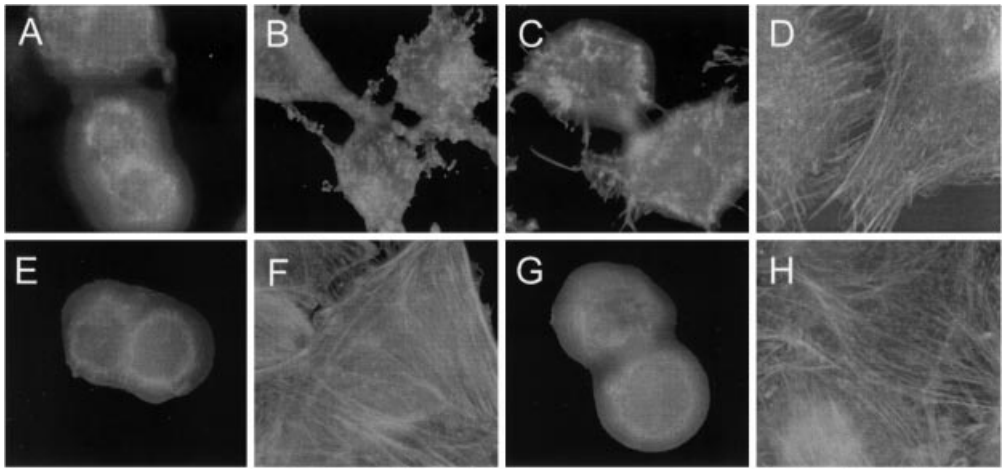

Figure 4 Disruption of actin microfilaments by ExoS

Hela cells were infected with bacteria expressing ExoS (**A** and **E**), ExoS(E381A) (**B** and **F**), ExoS(Δ98-232) (**C** and **G**) or uninfected (**D** and **H**), followed by FITC–phalloidin staining of the actin microfilaments. Infection was allowed to continue for 1 h (**A**–**C**), and thereafter the cells were washed free of bacteria and incubated overnight with medium containing gentamicin (**E**–**H**).

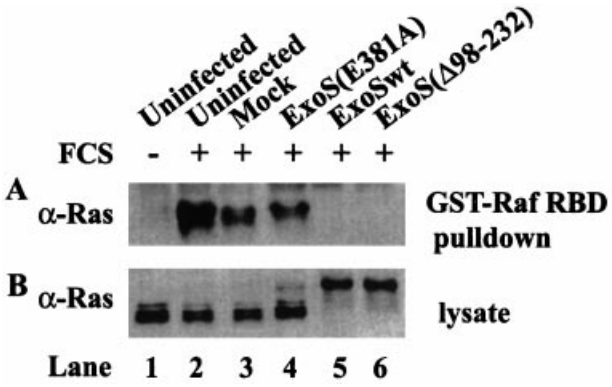

Figure 5 Affinity precipitation of active Ras with GST-Ras-BD of Raf-1

(**A**) Serum-starved HeLa cells were infected with wild-type and mutant ExoS proteins for 80 min prior to a 2 min treatment with 10% FCS (lanes 2–6) or not (lane 1). Lysates were subjected to affinity precipitation with GST-Raf-Ras-BD (all lanes). Ras proteins were detected by immunoblotting with monoclonal anti-Ras antibodies; lanes 1 and 2, uninfected HeLa lysates; lane 3, mock infection; lane 4, infection with ExoS(E381A); lane 5, infection with wild-type (wt) ExoS; lane 6, infection with ExoS(Δ98-232). (**B**) Whole cell lysates from cells infected as described above, separated by SDS/PAGE, and Ras proteins detected by immunoblotting with anti-Ras antibodies.

bacteria and incubated overnight with medium containing gentamicin. We observed that cells transiently infected with the ADP-ribosylation mutant ExoS(E381A) recovered their original cell structure and morphology overnight (Figure 4, compare panel F with panels H and B), suggesting that infection with ExoS(E381A), which contains GAP activity and dramatically reduced ADP-ribosylation activity, results in a reversible phenotype. In contrast, cells infected with either wild-type ExoS or ExoS(Δ98-232) for 1 h, washed and left overnight to recover, were profoundly different in their appearance (Figure 4, compare panels E and G with panels F and H). These cells exhibited a sustained changed morphology, they were semi-detached from the Petri dish and were not healthy. Both the wild-type ExoS and ExoS(Δ98-232) proteins harbour the ADP-ribosylation domain and the putative activity towards GTPase-defined target protein(s). Thus, via an as yet uncharacterized target, the ADP-ribosylation domain of ExoS appears to mediate non-reversible disruption of actin microfilaments. Thus these results provide strong evidence for a cellular target for the ADP-ribosylation activity of ExoS upon infection.

ExoS infection modifies Ras GTPase activation *in vivo*

The C-terminus of ExoS ADP-ribosylates Ras *in vivo* and inhibits GTP loading and activation of Ras and its ability to interact with Raf upon stimulation with growth factors [19]. Previous work has also shown that the N-terminal GAP domain of ExoS, excluding the ADP-ribosylation domain, can inactivate Rho, Rac and Cdc42 *in vitro* [20]. To extend and test directly whether ExoS modification modulates the activity of other small GTPases, pull-down assays were employed using specific downstream substrate proteins for the various GTPases under investigation. As we have detected ADP-ribosylation of several members of the Ras family members *in vivo* (see above), we wished to examine any changes in *in vivo* activity status of a selection of these small GTPases. Those chosen for further *in vivo* studies were Ras, Ral, RhoA, Cdc42, Rac1, Rap1 and Rap2.

The small GTPase Ras plays a critical role in activation of several downstream cascades, which are involved in both regulated and de-regulated cell proliferation, as well as the control of differentiation and progression through the G_1 phase. In addition, Ras has been implicated in cell survival [27,28]. HeLa cells were infected with *Y. pseudotuberculosis* expressing wild-type ExoS, ExoS(E381A) or ExoS(Δ98-232) [19]. We have shown that wild-type and mutant proteins are equally and efficiently expressed and translocated into HeLa cells [6,19,49,50]. Ras activation was measured *in vivo* by GST-Raf-Ras-BD pull-down assays. Treatment of uninfected cells with fetal calf serum (FCS) increased the affinity precipitation of Ras by GST-Raf-Ras-BD, reflecting Ras activation (Figure 5A, lane 2). No co-precipitation of Ras with GST-Raf-Ras-BD upon FCS stimulation was observed after infection with bacteria expressing wild-type ExoS

or ExoS(Δ98-232) (Figure 5A, lanes 5 and 6). Conversely, co-precipitation of Ras protein with GST-Raf-Ras-BD was detected upon stimulation with FCS after infection with ExoS(E381A) (Figure 5A, lane 4). Thus ExoS ADP-ribosylates Ras *in vivo* (Figure 5B, compare lanes 5 and 6 with 3 and 4) and inhibits GTP loading and activation of Ras and its ability to interact with Raf upon stimulation with growth factors, as we have reported previously upon stimulation with epidermal growth factor [19].

Ral GTPase is down-regulated by the ADP-ribosylation domain of ExoS

The Ras-like family of small GTPases includes, among others, Ras, Rap1, R-Ras and Ral. These proteins are classified in this group based upon similarities in the effector domain [27,28]. Similar to Ras, Ral proteins are active when GTP-bound. The exchange is catalysed *in vivo* by the Ral guanine nucleotide exchange factors (Ral-GEFs) [51]. Observations that mitogen-dependent activation of Ral proteins requires Ras activation have led to the suggestion that Ral-GEFs are Ras effector proteins, and that Ral is required for Ras-induced oncogenic growth, morphological transformation and induction of DNA synthesis [27,52–54]. Recently it has been suggested that the Ras/Ral pathway might affect the activity of the Forkhead box (FOXO4) transcription factor [55]. This suggests that Ral might be an important mediator of Ras-induced proliferation and survival signals, although the direct signal transduction pathways downstream of Ral and its effector Ral-binding protein 1, which associates with Ral in a GTP-dependent manner, have not been resolved [55–57]. We have already established that infection with ExoS down-regulates Ras [19], and since Ral is suggested to be a downstream target of Ras upon growth factor stimulation we wished to investigate the activity of Ral using a Ras-independent pathway. Calcium can activate Ral independently of Ras [41,58]. To monitor the activation of Ral, we employed pull-downs with the Ral-BD of RalBP1 [41]. Treatment of uninfected cells with ionomycin increased the affinity precipitation of Ral by GST-RalBP1-Ral-BD (Figure 6A, lanes 1 and 3). No co-precipitation of Ras with GST-Raf-Ras-BD upon ionomycin treatment was observed (Figure 6C, lanes 1 and 3). On the other hand, both Ras and Ral could be affinity precipitated upon stimulation with FCS using GST-Raf-Ras-BD and GST-RalBP1-Ral-BD, respectively (compare Figures 6A and 6C, lanes 1, 2 and 3). The activation of Ral reached maximal levels after 2 min stimulation with ionomycin (results not shown). After infection with ExoS or ExoS(Δ98-232) for 80 min, followed by stimulation with ionomycin, we could observe a clear ADP-ribosylation of Ral and Ras *in vivo* (Figures 6B and 6D, lanes 6 and 7). However, no co-precipitation of Ral proteins was detected employing the GST-RalBP1-Ral-BD pull-down technique (Figure 6A, compare lane 3 with lanes 6 and 7). Infection with ExoS(E381A), prior to stimulation with ionomycin, does not abrogate Ral protein activation and resulting precipitation with GST-RalBP1-BD, suggesting that ExoS does not increase the intrinsic GAP activity of Ral-GTP. Thus ExoS appears to inhibit Ral-mediated signal transduction via ADP-ribosylation *in vivo*.

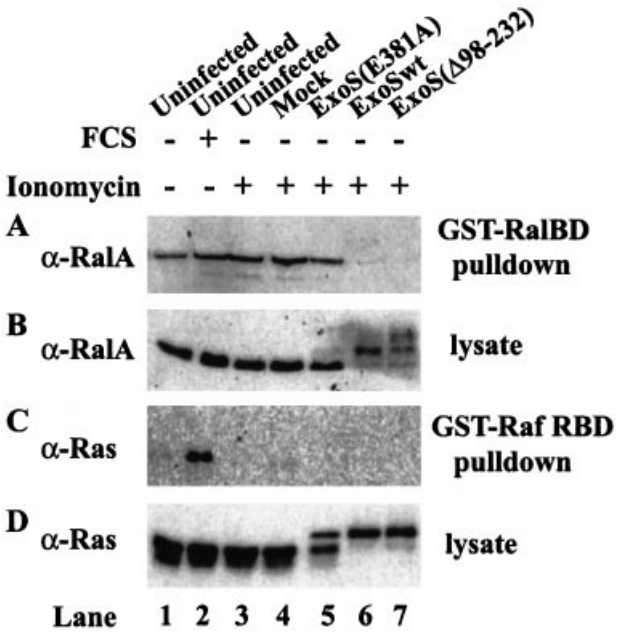

Figure 6 Affinity precipitation of active Ral with GST-Ral-BD of RalBP1

Serum-starved HeLa cells were infected for 80 min with *Yersinia* expressing different variants of ExoS, as indicated, and were treated with 10% FCS for 2 min (lane 2) or ionomycin (1 μg/ml) for 1 min (lanes 3–7) or were untreated (lane 1). (**A**) Lysates were subjected to affinity precipitation with GST-RalBP1-Ral-BD (all lanes) for 1 h. Ral proteins were detected by immunoblotting with monoclonal anti-Ral antibodies. (**B**) Whole cell lysates from (**A**) were separated by SDS/PAGE and Ral proteins were detected by immunoblotting with anti-Ral antibodies. (**C**) Lysates were subjected to affinity precipitation with GST-Raf-Ras-BD for 1 h. Ras proteins were detected by immunoblotting with monoclonal anti-Ras antibodies. (**D**) Whole cell lysates from cells infected as described were separated by SDS/PAGE and Ras proteins were detected by immunoblotting with anti-Ras antibodies. Lanes 1–3, uninfected HeLa cell lysates; lane 4, mock infection; lane 5, infection with ExoS(E381A); lane 6, infection with ExoS wild-type (wt); lane 7, infection with ExoS(Δ98-232).

Modulation of Rap1 and Rap2 by ExoS *in vivo*

The small GTPase Rap1 can inhibit Ras transformation by binding to downstream effectors of Ras signalling, but has lately also been suggested to be involved in a variety of biological processes, such as control of cell morphology, adherens junction positioning and cell adhesion [27,59,60]. Rap2 is also a member of the Ras-like family of GTPases and exhibits 60% identity with Rap1, but the function and regulation of Rap2 are unknown [27,28]. We analysed Rap1 and Rap2 activity *in vivo* in response to ExoS infection. To study Rap activities *in vivo*, the activation has to be carefully considered to ensure that analyses are not influenced by activation of Ras. Rap1 activation can be observed by an increase of intracellular cAMP, which activates the Rap1 and Rap2 specific guanine exchange factor, named Epac [27,38,61]. In Figure 7 we show that a combination of forskolin and IBMX activates both Rap1 and Rap2 readily without a notable activation of Ras, although upon stimulation with FCS all three GTPases, Ras, Rap1 and Rap2, were activated (Figures 7A, 7C and 7E, compare lanes 1, 2 and 3). Whole cell lysate from cells that have been infected with ExoS wild-type and ExoS(Δ98-232), both of which contain the ADP-ribosyltransferase domain, were able to modify both Rap1 and Rap2, whereas ExoS(E381A) showed no ADP-ribosylation activity towards Rap1 and Rap2 (Figures 7B, 7D and 7F, compare lanes 1, 2 and 5 with lanes 6 and 7). We monitored activity of both endogenous Rap1 and Rap2 using pull-downs with the Rap downstream target, GST-RalGDS-Rap1-Rap-BD, after infection with ExoS and ExoS(Δ98-232) (Figures 7A and 7C, lanes 6 and 7). Cells infected with the ribosylation-negative ExoS(E381A) mutant showed reduced Rap1 activation. A significant reduction was not observed with Rap2, indicating that the GAP domain of ExoS is a functional Rap1 GAP, but does not appear to function as a GAP for Rap2 (Figures 7A and 7C, compare lanes 4 and 5). In

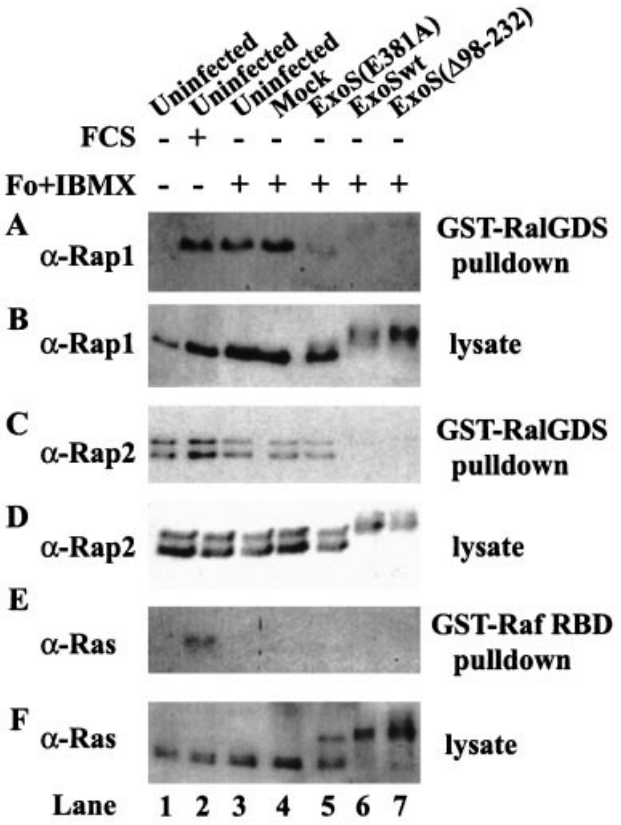

Figure 7 Precipitation of active Rap1/2 with GST-Rap-BD of RalGDS

Serum-starved HeLa cells were infected for 80 min with *Yersinia* expressing different variants of ExoS and were treated with 10% FCS for 2 min (lane 2) or forskolin (Fo; 10 μM) + IBMX (100 μM) for 10 min (lanes 3–7), or were untreated (lane 1). (**A**) Lysates were subjected to affinity precipitation with GST-RalGDS-Rap1/2 for 1 h (all lanes). Rap1 proteins were detected by immunoblotting with monoclonal anti-Rap1 antibodies. (**B**) Whole cell lysates from (**A**) were separated by SDS/PAGE and Rap1 proteins were detected by immunoblotting with anti-Rap1 antibodies. (**C**) The membrane from (**A**) was reprobed with monoclonal anti-Rap2 antibodies. (**D**) The membrane from (**B**) was reprobed with monoclonal anti-Rap2 antibodies. (**E**) Lysates were subjected to affinity precipitation with GST-Raf-Ras-BD for 1 h (all lanes). Ras proteins were detected by immunoblotting with monoclonal anti-Ras antibodies. (**F**) Whole cell lysates from (**E**) were separated by SDS/PAGE and Ras proteins were detected by immunoblotting with anti-Ras antibodies. Lanes 1–3, uninfected HeLa cell lysates; lane 4, mock infection; lane 5, infection with ExoS(E381A); lane 6, infection with ExoS wild-type; lane 7, infection with ExoS(Δ98-232).

order to establish further that ExoS(E381A) does not target Rap2 with its GAP domain, we extended our analysis to cells transiently transfected with haemagglutinin-tagged Rap2 in the presence or absence of haemagglutinin-tagged-PDZ-GEF, the exchange factor for Rap1. As we observed previously, infection with ExoS modifies the transiently transfected haemagglutinin-tagged Rap2, and we observed a decreased binding of haemagglutinin-tagged Rap2 with GST-RalGDS-Rap-BD, but not after infection with ExoS(E381A), similar to that observed above with the endogenous proteins (results not shown). Thus, both Rap1 and Rap2 are modified by ExoS. ADP-ribosylation of Rap1 and Rap2 results in decreased activity, as measured by interaction with the downstream target, GST-RalGDS-Rap-BD. In addition, ExoS also appears to function as a GAP for Rap1 *in vivo*, which was not observed for Rap2. Thus ExoS shows differential GAP activity towards Rap1 and Rap2.

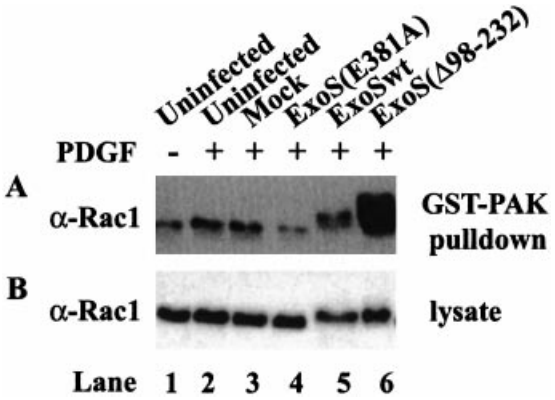

Figure 8 Precipitation of active Rac with GST-Rac-BD of PAK

Serum-starved HeLa cells were infected for 80 min with *Yersinia* expressing different variants of ExoS or ExoS wild-type and were treated with PDGF (3 ng/ml) for 2 min (lanes 2–6) or were untreated (lane 1). (**A**) Lysates were subjected to affinity precipitation with GST-PAK-Rac-BD for 30 min (all lanes). Rac proteins were detected by immunoblotting with monoclonal anti-Rac antibodies. (**B**) Whole cell lysates from cells infected as described were separated by SDS/PAGE and Rac proteins were detected by immunoblotting with anti-Rac antibodies. Lanes 1 and 2, uninfected HeLa cell lysates; lane 3, mock infection; lane 4, infection with ExoS(E381A); lane 5, infection with ExoS wild-type; lane 6, infection with ExoS(Δ98-232).

GAP-deficient ExoS protein activates Rac

The small GTPases of the Rho subfamily are key signal transducers, associated with cytoskeletal reorganization. The Rho subfamily proteins are suggested to be important in several cellular processes, such as cell shape determination, migration, transcriptional regulation and vesicle trafficking [29,30]. Rho proteins are targets for several toxins that covalently modify Rho, such as C3, which inactivates Rho by ADP-ribosylation of Arg-41 [62,63]. Toxins A and B from *Clostridium difficile* inactivate Rho by monoglycosylation at Thr-37, but also Rac and Cdc42 [64,65]. Cytotoxic necrotizing factor, conversely, activates Rho, Rac and Cdc42 by deamidation of Gln-63 [66,67]. Several other toxins have been suggested to regulate the active versus the inactive state of Rho, including ExoS, ExoT, SptP and YopE, which act as GAP proteins for several members of the Rho family *in vitro* [12,20,68–72]. However, the *in vivo* activity of these GAPs has never been addressed. Pull-down assays for determining levels of active GTP-bound Rho, Rac and Cdc42 in cells using recombinant domains of target proteins (PAK for Rac, WASP for Cdc42 and Rhotekin for Rho) have become a rapid and central tool to establish the GTP/GDP ratio, i.e. the active/inactive state, of Rho, Rac and/or Cdc42 [39,40,73].

We have employed PDGF stimulation to activate Rac in HeLa cells (Figure 8, compare lanes 1 and 2). The same variants of ExoS proteins were employed as above. Cells infected with the ADP-ribosylation-negative ExoS(E381A) mutant, followed by stimulation with PDGF, showed a reduced Rac activation as measured by GST-PAK-Rac-BD pull-downs (Figure 8, compare lanes 4 with 1). This indicates that the ExoS GAP domain has the capacity to function as a Rac-GAP *in vivo*, as predicted from the *in vitro* analysis. Unexpectedly, we observed that cells infected with the wild-type ExoS followed by stimulation with PDGF showed a similar or slightly increased ability to interact with GST-PAK-Rac-BD compared with uninfected cells, and the Rac proteins that were precipitated were modified (Figure 8, lanes 5). Thus, full-length ExoS appears, if anything, to activate Rac *in*

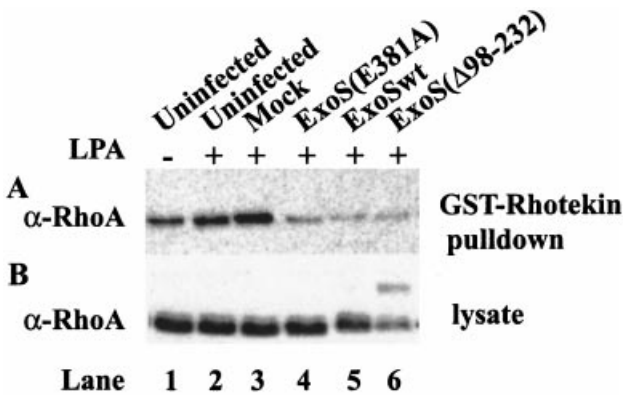

Figure 9 Regulation of Rho-GTPase activity by ExoS GAP domain

HeLa cells were infected for 80 min with *Yersinia* expressing different variants of ExoS and were treated with LPA (1 μg/ml) for 1 min (lanes 2–6) or were untreated (lane 1). (**A**) Lysates were subjected to affinity precipitation with GST-Rhotekin-Rho-BD for 45 min (all lanes). Rho proteins were detected by immunoblotting with monoclonal anti-RhoA antibodies. (**B**) Whole cell lysates from cells infected as described were separated by SDS/PAGE and Rho proteins were detected by immunoblotting with anti-RhoA antibodies. Lanes 1 and 2, uninfected HeLa cell lysates; lane 3, mock infection; lane 4, infection with ExoS(E381A); lane 5, infection with ExoS wild-type (wt); lane 6, infection with ExoS(Δ98-232).

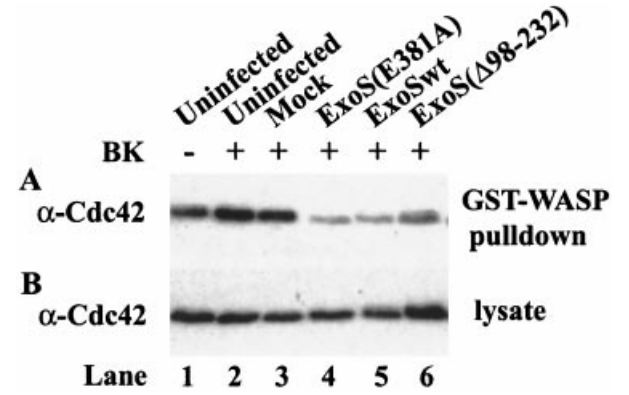

Figure 10 Affinity precipitation of active Cdc42 with GST-Cdc42-BD of WASP

Serum-starved HeLa cells were infected for 80 min with *Yersinia* expressing different variants of ExoS and were treated with bradykinin (BK; 4.1 μM) for 5 min (lanes 2–6) or were untreated (lane 1). (**A**) Lysates were subjected to affinity precipitation with GST-WASP-Cdc42-BD for 30 min (all lanes). Cdc42 proteins were detected by immunoblotting with monoclonal anti-Cdc42 antibodies. (**B**) Whole cell lysates from cells infected as described were separated by SDS/PAGE and Cdc42 proteins were detected by immunoblotting with anti-Cdc42 antibodies. Lanes 1 and 2, uninfected HeLa cell lysates; lane 3, mock infection; lane 4, infection with ExoS(E381A); lane 5, infection with ExoS wild-type (wt); lane 6, infection with ExoS(Δ98-232).

vivo. Furthermore, and even more surprisingly, in cells infected with ExoS(Δ98-232), which contains the ADP-ribosylation domain of ExoS but lacks the GAP domain, we observed a dramatic increase of Rac in a GTP-bound state upon stimulation with PDGF compared with the wild-type ExoS (Figure 8, compare lanes 6 with 7). Thus loss of the N-terminal GAP domain of ExoS results in an ExoS, which activates the small GTP-binding protein Rac. From our analysis of Rac activity *in vivo* it is clear that ExoS is able to ADP-ribosylate Rac; however, this modification does not appear to modify significantly Rac activity *in vivo*. Furthermore, it is clear that alone the ADP-ribosylation domain of ExoS acts as a robust Rac activator.

ExoS is a functional Rho-GAP *in vivo*

RhoA proteins can be activated readily by stimulation with LPA [40,74]. HeLa cells infected with the ADP-ribosylation-negative ExoS(E381A) mutant, followed by stimulation with LPA, show a reduced Rho activation, as measured by GST-Rhotekin-Rho-BD pull-downs (Figure 9, compare lanes 4 with 1). These results indicate that the GAP domain of ExoS is a functional Rho-GAP *in vivo*. Similar to ExoS(E381A), infection with wild-type ExoS followed by stimulation with LPA also inhibits endogenous RhoA activation (Figure 9, lanes 5). Infection with ExoS(Δ98-232), which contains only the ADP-ribosylation domain, also inhibits Rho activation (Figure 9, lanes 6). This may be due to the higher ADP-ribosylation activity of this deletion mutant as compared with ExoS (Figure 9, lanes 5 and 6), since an ADP-ribosylated RhoA protein can be observed in the whole cell lysate after infection for 80 min. Similar to our analysis of Rac, above, this suggests that great care should be taken when interpreting results achieved with deletion variants of ExoS. In conclusion, ExoS appears to act as a GAP for Rho *in vivo*, while the contribution of ADP-ribosylation to this modulation of Rho activity *in vivo* appears to be slight, if significant at all.

ExoS acts as a GAP for Cdc42 *in vivo*

Cdc42 can be activated by stimulation with bradykinin and, similar to microinjection of activated Cdc42, filapodial protrusions can be observed [73,75]. Cells infected with the ExoS(E381A) mutant, followed by stimulation with bradykinin, show a clear reduction of Cdc42 activation, as measured by GST-WASP-Cdc42-BD precipitation (Figure 10, compare lanes 4 with 1). A significant proportion of the Cdc42 protein pool appears to be down-regulated by both ExoS(E381A) and wild-type ExoS, thus indicating that the GAP domain of ExoS is a functional Cdc42 GAP. These observations are in agreement with the *in vitro* observations, which have suggested that ExoS contains a GAP activity against Cdc42 [20]. Similar to our results with RhoA above, the ADP-ribosylation domain of ExoS, lacking the GAP domain, also impairs the precipitation of Cdc42 with the GST-WASP-Cdc42-BD mutant.

DISCUSSION

In this report we have focused on two analyses. Firstly, we wanted to ask which of the small GTPases are modified *in vivo* by ADP-ribosylation, specifically by the toxins ExoS and ExoT of *P. aeruginosa*. Secondly, after identifying multiple endogenous small GTP-binding proteins as *in vivo* ribosylation targets for ExoS, we asked if these modifications resulted in a modulation of small GTPase activity *in vivo*. In our studies we have employed a clinical isolate of *P. aeruginosa* as well as genetically engineered *Y. pseudotuberculosis* strains to deliver ExoS proteins into eukaryotic cell cytosol [6,10]. The latter type III secretion system allows direct measurements of the consequence of individual type III translocated cytotoxins on cell physiology ([6] and references therein). We have investigated two bifunctional toxins, ExoS and ExoT. *In vitro* studies have shown that both toxins contain GAP activity together with a ADP-ribosyltransferase domain. Our initial analysis employed recombinant ExoS protein *in vitro*, which should provide an initial indication of ADP-ribosylation observed as an increase in protein mass due to the ADP-ribosylation of small GTPases. Secondly, we have examined ADP-ribosylation *in vivo* by the shift in protein mass

Table 1 Summary of *in vitro* and *in vivo* modification of GTPases by ExoS and ExoT

+ indicates GTPase modification; — indicates no GTPase modification observed. ND, not determined; *Ps*, *P. aeruginosa*; *Yer*, *Y. pseudotuberculosis*.

	ExoS ADP-ribosylation activity			ExoT ADP-ribosylation activity	
GTPase	*In vitro*	*In vivo* (*Ps*)	*In vivo* (*Yer*)	*In vivo* (*Ps*)	*In vivo* (*Yer*)
Ras	+	+	+	—	—
N-Ras	ND	+	+	—	—
K-Ras	ND	+	+	—	—
R-Ras	ND	+	+	—	—
Rap1	+	+	+	—	—
Rap2	ND	+	+	—	—
RalA	+	+	+	—	—
TC21	ND	—	—	—	—
RhoA	+	—	—	—	—
RhoB	+	—	—	—	—
RhoD	+	—	—	—	—
Rac1	+	+	+	—	—
Cdc42	+	+	+	—	—
Rab4	+	—	—	—	—
Rab5	+	+	+	—	—
Rab8	ND	+	+	—	—
Rab11	ND	+	+	—	—
Rab27	ND	—	—	—	—
Ran	ND	—	—	—	—
ARF3	ND	—	—	—	—

Table 2 Summary of activation status of selected GTPases *in vivo* after ExoS modification, using the pull-down assay

GTPase	ExoS GAP activity	ExoS ADP-ribosylation activity
Ras	No	Yes
Ral	No	Yes
Rap1	Yes	Yes
Rap2	No	Yes
Rac1	No	No
RhoA	Yes	Yes/No
Cdc42	Yes	Yes/No

of endogenous small GTP-binding proteins following infection by bacteria expressing ExoS and ExoT.

These results, summarized in Table 1, show that ExoS displays a highly promiscuous ADP-ribosylation activity towards small GTPases, both *in vitro* and also *in vivo*. All tested GTP-binding proteins *in vitro* show a slower mobility after incubation with ExoS, thus ExoS is able to modify all GTPases tested *in vitro* [15,76]. Upon infection with ExoS *in vivo* the effect of ExoS on the different Ras-related proteins was more restricted; we observed that H-Ras, N-Ras, K-Ras, R-Ras, Rap1, Rap2, Rab5, Rab8, Rab11, RalA, Cdc42 and Rac1 are all ADP-ribosylated by ExoS *in vivo*. However, we were unable to observe modification of TC21, Ran, RhoA, RhoB, ARF3, Rab4 or Rab27. In contrast, ExoT shows no detectable ADP-ribosylating activity towards any of the GTPases tested *in vivo*, regardless of whether ExoT protein is secreted and translocated by the type III system of *P. aeruginosa* or by *Y. pseudotuberculosis*. Since a strain of *P. aeruginosa* deleted for ExoS has no detectable *in vivo* small G-protein ADP-ribosylation activity and ExoT has no identifiable ADP-ribosylation activity against the GTPases tested here, we suggest that ExoS is the major ADP-ribosyltransferase protein in *P. aeruginosa* towards the small GTPases. One is then left to consider the target of the putative *in vivo* ADP-ribosylation activity of ExoT on cellular infection by *P. aeruginosa*. Interestingly, in our previous studies [12], we observed that ExoT(R149K), which is deficient in GAP activity, is still able to cause a morphological change in infected HeLa cells. This is of importance, since it suggests that the ADP-ribosylating activity of ExoT targets another, as yet unidentified, host protein that is distinct from the Ras family members examined here. It should be emphasized that our observations of ADP-ribosylation activity by ExoS on *in vivo* substrates are identical using two different systems, with both *Pseudomonas* and *Yersinia* strains. Our observations are strengthened since they are in agreement with Olson and co-workers [77], who have compared two-dimensional electrophoresis of substrate modifications by ExoS *in vitro* with that following bacterial translocation in cells. It has been reported by Vidal and co-workers [16] that ExoS ADP-ribosylation of Rab4 in permeabilized reticulocytes affects Rab4's function in membrane recycling, but the mechanism behind this inhibition is unclear [16].

In vivo studies with ExoS have shown that the expression of ExoS affects cell viability [6], and to date this effect on viability has been considered most probably as a result of ADP-ribosylation of Ras [19], which has a central role in cell signalling. We wished to examine changes in *in vivo* activity status of a selection of the small GTPases, which we had identified as the ExoS ADP-ribosylation substrates *in vivo*. To extend and directly test whether ExoS modification modulates the activity of these small GTPases, pull-down assays using specific downstream substrate proteins for the various GTPases under investigation were employed (summarized in Table 2). As we have reported previously [19], ExoS ADP-ribosylates Ras *in vivo* and inhibits GTP loading and activation of Ras and its ability to interact with Raf upon stimulation with growth factors. Bacterially secreted ExoS *in vivo* shows no GAP activity towards endogenous Ras, in agreement with *in vitro* reports [20].

We also studied the modulation of Ral activity by ExoS. It has recently been proposed that Ras/Ral pathway might affect the activity of further downstream transcription factors, such as Forkhead box (FOXO4) [55]. This suggests that Ral might be an important mediator in proliferation and survival, although the direct signal transduction pathways downstream of Ral have not been resolved [55–57]. We observed a clear ADP-ribosylation activity towards Ral *in vivo* with both *Pseudomonas* and *Yersinia* strains (which exclusively express ExoS). Furthermore, Ral activity was inhibited after ExoS infection. Since the ADP-ribosylation-deficient ExoS mutant did not appear to affect Ral activity, we presume that ExoS does not act as an efficient GAP for Ral *in vivo*. Thus, ExoS appears to inhibit Ral-mediated signal transduction via ADP-ribosylation *in vivo*. While the molecular mechanism behind this inhibition is unclear, it is possible that the ADP-ribosyl modification of Ral hinders the interaction between Ral and RalBP1, although it is also plausible that the modification hampers the postulated exchange activity for Ral [17]. Further *in vivo* studies of this matter are required.

Rap1 and Rap2 are also members of the Ras-like family of GTPases and exhibit high homology to Ras in the effector domain [27,28]. We explored the modification of Rap1 and Rap2 activity *in vivo* in response to ExoS infection. The modification of Rap proteins by ExoS is a controversial area, since it has previously been reported that Rap1 is not modified *in vivo* by ExoS [43]. However, we see a clear modification of Rap proteins by ExoS under conditions sufficient for Ras modification, in agreement with recent report of others (see above and [48,77]). Infection with full-length ExoS results in the ADP-ribosylation

of Rap1 and Rap2 and the inhibition of Rap1 and Rap2 activation as measured by pull-down assay. Furthermore, the GAP domain of ExoS shows significant GAP activity towards Rap1 *in vivo*. In our hands, however, Rap2 does not appear to be a significant target for ExoS GAP activity.

Several toxins have been shown to regulate the nucleotide state of Rho subfamily members and they act as GAP proteins for several members of the Rho family *in vitro* [12,20,68–72]. However, the *in vivo* GAP activity of toxins, such as ExoS, ExoT, SptP and YopE, has never been reported. Incorporation of GAP activities into this family of bacterial toxins offers a great advantage in the anti-phagocytic strategy during infection, and is further illustrated by the fact that all of these effectors proteins have been found to prevent bacterial internalization [6,14,69,78]. We have investigated the *in vivo* modulation of RhoA, Rac and Cdc42 after infection of ExoS. Cells infected with ADP-ribosylation-negative ExoS mutant show reduced Rac activation, as measured by pull-down assay. This indicates that the ExoS GAP domain functions as a Rac-GAP *in vivo*, as predicted from earlier *in vitro* results [20]. However, we observed that cells infected with wild-type ExoS showed a similar or slightly increased ability to interact with GST-PAK-Rac-BD, which was unexpected. Also surprising was the fact that the effector domain of ADP-ribosylated Rac was still able to interact with GST-PAK-Rac-BD. Furthermore, cells infected with a GAP-deficient ExoS, which contains a complete ADP-ribosylation domain of ExoS, showed a dramatic increase of Rac in a GTP-bound state, resulting in a robust activation of the small GTPase Rac. From our analysis of Rac activity *in vivo* it is clear that ExoS is able to ADP-ribosylate Rac; however, ADP-ribosylation in the context of the full-length ExoS molecule does not appear to modulate Rac activity significantly *in vivo*, either negatively or positively. Loss of ExoS ADP-ribosylation activity (through mutation of the ADP-ribosylation domain) unveils a Rac GAP activity and subsequent Rac inactivation. Conversely, loss of GAP activity (through deletion of the GAP domain) in ExoS reveals a capacity of the ExoS ADP-ribosylation domain to activate potently Rac *in vivo*. This experiment in particular highlights a drawback with using deletion variants of ExoS. The above results from two ExoS proteins, which are variants of the bifunctional enzyme ExoS, should be compared with the wild-type ExoS protein, which does not significantly modulate Rac activity *in vivo*, though it is clearly able to ADP-ribosylate Rac. Possible explanations for these results are that the ADP-ribosylation of Rac by ExoS hampers the intrinsic Rac GTP-to-GDP hydrolysis. Another possibility is that the ADP-ribosyl modification may change the protein conformation of Rac in such a way that it neutralizes the GAP domain of ExoS. Alternatively, auto-ADP-ribosylation of ExoS mediates a GAP domain that is a poor activator of the intrinsic hydrolysis of Rac, which has been suggested *in vitro* [79]. Finally, it is also possible that ribosylation of Rac by ExoS increases its activity for PAK independent of nucleotide loading. These results indicate that the mechanism of ExoS action is complex, and is likely influenced by its bifunctional mechanism of action. This experiment shows that deletion variants of proteins may not precisely reflect the *in vivo* situation when bacteria translocate toxins to the eukaryotic cell. In agreement with earlier *in vitro* reports, we observe that ExoS is also a functional Rho-GAP *in vivo*. It should be noted that Rho activity is still observed after infection with ExoS, thus indicating that the GAP domain of ExoS does not down-regulate the entire pool of endogenous Rho proteins. Thus, ExoS appears to act as a GAP for Rho *in vivo*, while the ADP-ribosylation is also able to contribute to the modulation of Rho activity *in vivo*, although the relevance of this activity physiologically is unclear, since at later times of infection cells are no longer viable. Finally, cells infected with wild-type ExoS show a clear reduction of Cdc42 activation. A significant proportion of the Cdc42 protein pool appears to be down-regulated, indicating that the GAP domain of ExoS is a functional and fairly efficient Cdc42 GAP. These observations are in agreement with *in vitro* observations, which have suggested that ExoS contains a GAP activity against Cdc42 [20]. However, Cdc42 is also inactivated by infection with a GAP-defective ExoS.

We have investigated seven selected small GTPases for *in vivo* activities and their ability to interact with downstream targets, which affect downstream signalling pathways important for DNA synthesis, morphology, adhesion and microbial invasion, and survival (Table 2). From our analyses we observe that Ras, Ral and both Rap1 and Rap2 lose their ability to interact with downstream signalling proteins due to ADP-ribosylation activities after infection by wild-type ExoS. Cdc42 is modified by ExoS early after infection and ADP-ribosylation activities by ExoS clearly impair Cdc42's ability to interact with downstream targets. On the other hand, only Rap1, RhoA and Cdc42 were observed to be down-regulated by wild-type ExoS, through the GAP domain. Finally, we observed that the outcome after infection by wild-type ExoS on Rac1 is similar to stimulated uninfected cells, although Rac1 is ADP-ribosylated and has the ability to interact with the GST-PAK-Rac-BD. Further studies will define the importance of each individual modulation of different GTPases by ExoS and whether *P. aeruginosa* targets virulence proteins into the eukaryotic cytosol via a type III secretion-dependent mechanism as part of an anti-phagocytic strategy.

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