Ras effector pathway activation by epidermal growth factor is inhibited *in vivo* by exoenzyme S ADP-ribosylation of Ras

Maria L HENRIKSSON, Roland ROSQVIST, Maxim TELEPNEV, Hans WOLF-WATZ, and Bengt HALLBERG¹

Department of Cell and Molecular Biology, University of Umeå, S-901 87 Umeå, Sweden

We have examined the functional consequences of ADP-ribosyltransferase modification of Ras by the exoenzyme S (ExoS) protein of *Pseudomonas aeruginosa*. ExoS has been shown previously to ADP-ribosylate a number of proteins, including members of the Ras superfamily, which play an essential role in the processes of cell proliferation, differentiation, motility and cell division. HeLa and NIH3T3 cells were infected with ExoS protein, which was delivered via the type III secretion system of the heterologous host *Yersinia pseudotuberculosis*. Infection of mammalian cells with ExoS results in a change in the ratio of GTP/GDP bound directly to Ras *in vivo*. This ADP-ribosylation of Ras *in vivo* is mediated by the C-terminal domain of ExoS. Further, ExoS ADP-ribosylation of Ras *in vivo* inhibits activation of Ras and the ability to interact with the Ras binding domain of

Raf upon stimulation with epidermal growth factor (EGF). In the present study, we show that ExoS activity does not interfere with EGF receptor phosphorylation itself, nor with the formation of a Grb2-activated Shc complex upon EGF stimulation, consistent with ExoS blockage of this mitogenic signalling pathway at the level of Ras. This is further supported by our observation of a substantial inhibition of extracellular signal-regulated kinase and protein kinase B/Akt kinase activation in response to EGF upon ExoS infection. In conclusion, in the present study, the consequences of ExoS infection on Ras effector pathway *in vivo* have been defined.

Key words: ADP-ribosyltransferase, MAP kinase, PKB/Akt, *Pseudomonas aeruginosa*, Ras superfamily.

INTRODUCTION

Upon infection of eukaryotic cells by pathogenic bacteria, various bacterial toxins covalently modify certain members of the Ras superfamily, thereby disturbing the regulatory mechanisms of these GTPase proteins and leading to an aberrant signal transduction [1]. The Ras superfamily is involved in signal transduction pathways stimulated in response to a multitude of external factors, such as epidermal growth factor (EGF) [1a–5]. Upon EGF receptor stimulation, a complex between the adapter proteins Shc and Grb2 is formed recruiting the guanine nucleotide exchange factor Sos and leading to the activation of Ras [6,7]. Ras proteins are low-molecular mass G-proteins, which function as molecular ‘switches’, and are active when bound to GTP and inactive when bound to GDP [8,9]. Ras play a critical role in activation of the mitogen-activated protein (MAP) kinase cascade [10–12], which is involved in both regulated and deregulated cell proliferation, as well as the control of differentiation and progression through the G1-phase [13–16]. Furthermore, Ras have been implicated in cell survival, which is mediated through activation of phosphatidylinositol 3-kinase, which generates the second messenger PtdIns(3,4,5)P₃, an activator of the protein kinases, protein kinase B (PKB)/Akt and 3-phosphoinositide-dependent protein kinase 1 [17–19].

The bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen, which can cause life-threatening infections in immunocompromised hosts [20–22]. Recently it was reported that *Ps. aeruginosa* utilizes a type III secretion-dependent mechanism, similar to *Yersinia pseudotuberculosis*, which appears to be functionally conserved among several bacterial pathogens, for

secretion and translocation of virulence factors into the eukaryotic cytosol [23–26]. One of these virulence factors is exoenzyme S (ExoS), which belongs to the family of ADP-ribosylating toxins [27,28]. The results of several observations suggest that ExoS possesses more than one functional domain. First, intracellular expression of the N-terminal domain of ExoS, a domain with homology with a bacterial toxin from *Yersinia*, named *Yersinia* outer protein (YopE), elicits the disruption of actin microfilament structure through an, as yet, uncharacterized mechanism [26,29,30]. Secondly, the C-terminal domain has been shown to contain the ADP-ribosylating activity and is lethal to eukaryotic cells [26,31–33]. The catalytic activity of ExoS is known to be dependent on an eukaryotic host protein named factor activating ExoS (FAS) [34,35]. *In vitro*, ExoS has been shown to ADP-ribosylate a large number of proteins, including vimentin and members of the Ras family [36,37]. During the course of the study, Ganesan et al. [38] demonstrated that ExoS is able to ADP-ribosylate c-Ha-Ras *in vitro* at more than one site, with arginine-41 being the preferred site of ADP-ribosylation, and McGuffie et al. [39] observed that an ExoS producing strain, *Ps. aeruginosa* 388, modifies Ras. However, no studies have yet described the effect on the enzymic-signalling pathway mediated by Ras after ExoS modification *in vivo*. In the present study, the *Y. pseudotuberculosis* type III secretion system has been employed as a tool to deliver ExoS proteins into cells [25,26,29,40,41]. Thus it was demonstrated that the C-terminal portion of the ExoS protein modified Ras *in vivo*. Furthermore, modification of Ras by ExoS was able to significantly increase the amount of endogenous Ras in a GDP-bound state *in vivo*. Also affected was the ability of Ras to be activated by EGF-receptor stimulation,

Abbreviations used: ExoS, exoenzyme S; Erk, extracellular signal-regulated kinase; EGF, epidermal growth factor; GST, glutathione S-transferase; MAP, mitogen-activated protein; PKB, protein kinase B; YopE, *Yersinia* outer protein; RBD, Ras binding domain.

¹ To whom correspondence should be addressed (e-mail Bengt.Hallberg@cmb.umu.se).

an effect that, importantly, was not observed with a catalytically inactive ExoS mutant. Another consequence of ExoS modification of Ras was the abrogation of the Ras–Raf interaction normally observed upon growth factor stimulation. In addition, it was shown that ExoS activity did not interfere with EGF receptor phosphorylation, or with the formation of a Grb2-activated Shc complex upon EGF stimulation. Downstream of Ras, it was shown that ExoS-mediated modification of Ras inhibited the phosphorylation of the MAP kinase, extracellular signal-regulated kinase (Erk)1/2, as well as PKB/Akt kinase. In conclusion, we have demonstrated that the ExoS protein functions *in vivo* to perturb multiple effector signal transduction pathways.

EXPERIMENTAL

Bacterial strains, plasmids and antibodies

Y. pseudotuberculosis expressing wild type and a point mutated *Ps. aeruginosa* ExoS gene product, YPIII (pIB251, pTS103), and YPIII (pIB251, pTS106), cultivation and infection have been described previously [26]. Two deletion mutants, YPIII (pIB251, pTS103, Δ 98–232) and YPIII (pIB251, pTS106, Δ 98–232) were generated using standard plasmid manipulation procedures. The plasmids pTS103 and pTS106 were excised with the restriction enzyme *EagI* and religated, creating a deletion between bp 289 and 694. Plasmids were sequenced before being retransformed into YPIII (pIB251). H-Ras (259) agarose-conjugated, K-Ras (F234) and H-Ras (F235) antibodies were purchased from Santa Cruz (New York, NY, U.S.A.). Agarose-conjugated anti-phosphotyrosine 4G10 monoclonal anti-phosphotyrosine, polyclonal anti-human Shc and EGF receptor antibodies were obtained from UBI (Lake Placid, NY, U.S.A.). Monoclonal Ras (R02120), Shc and Grb2 (G16720) antibodies were obtained from Transduction Laboratories (Lexington, KY, U.S.A.). p44/42 MAP kinase, phospho-Akt (Ser⁴⁷³) and phospho-p44/42 MAP kinase (Thr²⁰²/Tyr²⁰⁴) antibodies were obtained from New England Biolabs (Beverly, MA, U.S.A.). EGF was from UBI. Glutathione S-transferase (GST)-fusion protein encompassing the Ras binding domain of Raf-1 was kindly provided by Dr J. Downward (Imperial Cancer Research Fund, London, U.K.).

Cell culture, cell lysis and immunoprecipitation

HeLa and NIH3T3 cells were grown in minimal essential medium and RPMI 1640 respectively, supplemented with 10% (v/v) fetal-bovine sera and 100 units/ml penicillin. Following bacterial infection (see above), at the times shown on the Figures, and stimulation with EGF (50 ng/ml) for 2 min, cells were washed twice in ice-cold PBS and 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 15000 *g* for 10 min at 4 °C. Similar volumes of lysate were incubated with primary antibody or GST-fusion proteins for 1 h and with Protein G-agarose or glutathione-agarose (Pharmacia) for a further 30 min. After four washes in lysis buffer, samples were boiled in SDS/PAGE sample buffer [42,43].

³²P-labelling of HeLa cells *in vivo*

Measurement of the nucleotide-bound state of Ras in HeLa and NIH3T3(V12, K-Ras) cells. HeLa cells were metabolically labelled over night with [³²P]_i (Amersham), as described previously [43–45]. Cells were then treated for various times with *Y. pseudotuberculosis* expressing wild-type ExoS or ExoS(E381A);

Glu³⁸¹ → Ala) proteins and stimulated with EGF (50 ng/ml) for 2 min before harvesting. Immunoprecipitations were carried out as described above, and immunoprecipitated samples were separated by PEI-cellulose thin-layer chromatography (Merck 15675 plates) and analysed by PhosphorImager (Molecular Dynamics).

ADP-ribosylation of Ras *in vitro*

The ADP-ribosylation assay *in vitro* was performed as described by Frithz-Lindsten et al. [26]. Briefly, 10 μ l of secreted ExoS/ExoS(E381A) were added to HeLa cell lysate and incubated with [³²P]NAD (Dupont-NEN, Stevenage, Herts, U.K.) for 1 h at 37 °C. The samples were then subjected to SDS/PAGE and immunoblotting.

RESULTS

ADP-ribosylation of Ras by bacterially expressed ExoS *in vivo*

The possibility that Ras was a target of ExoS *in vivo*, and whether this modification resulted in a change in an activation status of Ras was investigated. To achieve this, wild-type ExoS and a set of ExoS mutants, ExoS(E381A), ExoS(Δ 98–232) and ExoS(Δ 98–232, E381A) were employed (Figure 1A). It has been shown that ExoS ADP-ribosylates Ras *in vitro* [36,38] and that the ADP-ribosylation activity is associated with the C-terminal region of ExoS [26,31,33]. Amino acid residue 381 in the catalytic domain of ExoS [ExoS(E381A)] is essential for ADP-ribosyltransferase activity [26,32]. Two further mutants, ExoS(Δ 98–232) and ExoS(Δ 98–232, E381A), were constructed, based on the shared

Figure 1 Diagram of wild-type and other constructs of ExoS, expressed by *Y. pseudotuberculosis*, used for infection of either HeLa or NIH3T3 cells

(A) Schematic representation of ExoS mutants employed in this study. (i) Wild-type ExoS [YPIII (pIB251, pTS103)]; (ii) ExoS mutant harbouring a E381A mutation {ExoS(E381A) [YPIII (pIB251, pTS106)]}; (iii) ExoS mutant with deletion of amino acids 98–232 {ExoS(Δ 98–232) [YPIII (pIB251, pTS103, Δ 98–232)]}; (iv) ExoS(E381A) mutant with deletion of amino acids 98–232 {ExoS(E381A, Δ 98–232) [YPIII(pIB251, pTS106, Δ 98–232)]}. Important domains of ExoS, i.e. the catalytic ADP-ribosyltransferase domain [33] (stippled boxes) and the region of limited homology shared between ExoS and the *Yersinia* virulence factor YopE [29] (white boxes), are shown. (B) Western blot of whole cell extracts from HeLa cells. Cells were harvested 1 h after infection with the various ExoS constructs and were immunoblotted with anti-ExoS polyclonal antibody [26]. Lane 1, ExoS (wild type); lane 2, ExoS(Δ 98–232); lane 3, ExoS(E381A); lane 4, ExoS(E381A, Δ 98–232); lane 5: mock HeLa cell infection [YPIII (pIB251)]; lane 6, uninfected HeLa cells. * denotes a non-specific cross-reacting band. Molecular-mass markers (kDa) are shown on the right.

(A) HeLa cells were harvested after ExoS/ExoS(E381A) infection at the indicated time points. Whole-cell lysates were separated by SDS/PAGE, followed by immunoblotting with anti-Ras monoclonal antibody. **(B)** Endogenous Ras modification *in vitro* by bacterially expressed and purified ExoS (wt) or ExoS(E381A). HeLa cell lysates were incubated for 1 h at 37 °C in the presence of [³²P]NAD with purified ExoS/ExoS(E381A) or buffer control [26]. Samples were separated by SDS/PAGE and subjected to immunoblotting using anti-Ras antibody. **(C)** The membrane shown in **(B)** was stripped and analysed by autoradiography for detection of [³²P]NAD-labelled Ras protein. Molecular-mass markers are shown on the right.

HeLa and NIH3T3 (V-12, K-Ras) cells were uninfected (—) or infected for the indicated times with bacteria expressing ExoS (A), ExoS(E381A) (B) or which had been mock infected (M). Cells were stimulated, 2 min before harvesting, with EGF (+) at 50 ng/ml, as described in the Experimental section. Cells were then lysed, ³²P-labelled Ras protein was immunoprecipitated and the amount of GTP bound as a proportion of total guanine nucleotide bound was determined. Each time point represents two independent experiments performed in duplicate.

To assess Ras modification *in vivo*, HeLa cells, grown in 10 % fetal-calf serum, were infected for various times, harvested and the resultant lysates were separated on SDS/PAGE and immunoblotted with anti-Ras antibody (Figure 2A). After 15 min of infection with ExoS, a proportion of the total cellular Ras population showed slower mobility on SDS/PAGE. At 40 and 80 min post-infection all Ras proteins within the cell appeared to have been modified. At 80 min post-infection with ExoS all cells exhibited a changed morphology, characterized by disruption of actin filaments ([26]; R. Rosqvist, M. Telepnev and H. Wolf-Watz, unpublished work). This modification of Ras was not

observed with a bacterial strain expressing the ExoS(E381A) mutant. However, a small fraction of Ras proteins were modified after 80 min and, at this time point, cells showed signs of actin filament disruption (R. Rosqvist, M. Telepnev and H. Wolf-Watz, unpublished work). To investigate if the change in migration of Ras proteins observed (Figure 2A) was due to ExoS ADP ribosylation activity, HeLa cells were harvested and the lysate was incubated with [³²P]NAD and purified bacterially expressed wild-type ExoS protein or the ExoS(E381A) mutant protein for 60 min. The samples were then separated by SDS/PAGE before immunoblotting with an anti-Ras antibody (Figure 2B). A slower migrating form of Ras was visible when the HeLa cell lysate had been exposed to wild-type ExoS, which was not observed when the mutant ExoS(E381A) protein was used. The only incorporation of radiolabelled NAD observed was when non-infected cell lysate was mixed with wild-type ExoS protein (Figure 2C). Furthermore, infection of HeLa cells for 60 min with bacteria expressing the other mutants of ExoS showed that ExoS(A98-232), which contains a complete catalytic domain, modified Ras proteins as effectively as the wild-type ExoS protein. ExoS(A98-232, E381A), however, showed a similarly compromised ability to modify Ras proteins as the ExoS(E381A) (see Figure 4).

The finding that ExoS is capable of modifying Ras *in vitro* and *in vivo* raises the question whether ExoS is able to change the ratio of GTP/GDP bound to Ras *in vivo*, a reflection of the activation status of Ras within the cell itself, which can be determined by measuring the ratio of GTP (active) to GDP (inactive) bound to Ras. [³²P]P_i-labelled HeLa and NIH3T3(V12, K-Ras) cells were infected for various times with wild-type and mutant ExoS proteins, Ras was immunoprecipitated and the presence of labelled nucleotide was analysed by thin layer chromatography (Figure 3). ADP-ribosylation of Ras did not affect the efficiency of immunoprecipitation of Ras using the Y13-259 antibody (results not shown). Growing unstimulated HeLa cells, infected with bacteria expressing ExoS, showed a 2-fold decrease in the level of GTP-bound Ras, from 12 % to 5 % (two-sided $P < 0.001$), in a time-dependant manner over a period of 80 min (Figure 3). Stimulation of non-infected HeLa cells with

Figure 4 Affinity precipitation of active Ras with GST-Ras binding domain of Raf-1

(A) Serum-starved HeLa cells were infected with wild-type or mutant ExoS proteins for 60 min and were then treated with EGF (50 ng/ml) for 2 min (lanes 2–7), or were untreated (–) (lane 1). Lysates were subjected to affinity precipitation with GST–Raf–RBD (all lanes). Ras proteins were detected by immunoblotting with monoclonal anti-Ras antibodies. Lanes 1 and 2, uninfected HeLa cell lysates; lane 3, mock infection; lane 4, infection with ExoS(E381A, Δ98–232); lane 5, infection with ExoS(E381A); lane 6, infection with ExoS(Δ98–232); lane 7, infection with wild type ExoS. (B) Whole-cell lysates from cells infected as described in (A) were separated by SDS/PAGE and Ras proteins were detected by immunoblotting with anti-Ras antibodies. Molecular-mass markers are shown on the right.

EGF, for 2 min, caused a 6- to 7-fold elevation of the Ras–GTP level in HeLa cells. By 80 min after ExoS infection only 11 % of the cellular Ras was found in the GTP-bound state, a level similar to that observed in the non-stimulated and uninfected HeLa cells (Figure 3). In contrast, mock infection or infection of HeLa cells with the ExoS(E381A) mutant protein did not inhibit Ras–GTP loading in response to EGF (Figure 3). Furthermore, Ras immunoprecipitation from [32 P]P_i-labelled NIH3T3(V12, K-Ras) cells, after infection for 80 min with either ExoS, ExoS(E381A) or mock infected, resulted in no change in hydrolysis of GTP-bound Ras, compared with non-infected NIH3T3(V12, K-Ras) cells (Figure 3).

Further evidence for the inactivation of Ras proteins by ExoS *in vivo*, is the known specificity for the interaction of Ras–GTP with the Ras binding domain (RBD) of Raf-1 [14]. Lysates were prepared from HeLa cells, which had been infected with various ExoS mutants, and incubated with GST–Raf–RBD fusion protein immobilized on glutathione–Sepharose. After washing, bound proteins were eluted and subjected to SDS/PAGE and the gel was immunoblotted using pan anti-Ras antibodies (Figure 4). Treatment of uninfected cells with EGF increased the affinity precipitation of Ras by GST–Raf–RBD (Figure 4, compare lanes 1 and 2). No co-precipitation of Ras with GST–Raf–RBD upon EGF treatment was observed after infection with bacteria expressing wild-type ExoS and ExoS(Δ98–232), both of which retain full catalytic activity (Figure 4, compare lane 2 with lanes 6 and 7). Conversely, co-precipitation of Ras proteins with GST–Raf–RBD was detected upon EGF receptor stimulation after infection with either ExoS(E381A) or ExoS(Δ98–232, E381A) (compare lanes 2 and 3 with 4 and 5).

ExoS does not affect EGF-receptor phosphorylation or Shc–Grb2-activation complex formation

We wished to investigate the effect of ExoS on other components of the Ras signalling pathway. To assess whether the EGF receptor was phosphorylated after stimulation with EGF, HeLa cells were infected for various times with ExoS before being

Figure 5 Effect of ExoS infection on EGF receptor signalling components upstream of Ras

(A) and (B) ExoS infection does not inhibit EGF receptor autophosphorylation. After infection with ExoS for the indicated times, HeLa cells were treated with (+) or without (–) EGF for 2 min and then harvested. (A) The resulting lysates were subjected to SDS/PAGE and then immunoblotted with anti-phosphotyrosine antibody 4G10 (α-PY). (B) The membrane from (A) was stripped and reprobed with anti-EGF receptor antibody (α-EGFR). (C) Lysates were immunoprecipitated (IP) with anti-phosphotyrosine 4G10 antibodies and immunoblotted with anti-EGF receptor antibody (α-EGFR). (D) Effect of infection of ExoS and ExoS mutants on Shc phosphorylation. Tyrosine phosphorylation of Shc (SHC) was examined in (lane 1) unstimulated (–) and (lanes 2–7) EGF-stimulated (+) HeLa cells treated for 60 min as follows: lanes 1 and 2, uninfected HeLa cell lysates; lane 3, mock infection; lane 4, infection with ExoS(E381A, Δ98–232); lane 5, infection with ExoS(E381A); lane 6, infection with ExoS(Δ98–232); lane 7, infection with wild-type ExoS. Lysates were immunoprecipitated (IP) with anti-Shc antibodies (SHC) and immunoblotted with anti-phosphotyrosine antibody 4G10 (α-PY). (E) The membrane from (D) was stripped and reprobed with anti-Shc antibody (α-SHC). (F) Effect of ExoS infection on Shc–Grb2 complex formation. The membrane from (D) was probed with monoclonal Grb2 antibody (α-Grb2). Molecular-mass markers are shown on the right.

stimulated with EGF for 2 min and then lysed. The lysate proteins were separated and immunoblotted with the anti-phosphotyrosine antibody 4G10 (Figure 5A). Addition of EGF for 2 min resulted in a tyrosine phosphorylated protein of 170 kDa, which was not observed in non-stimulated cells. The membrane was stripped and reprobed with anti-EGF-receptor antibody, thus confirming that the band of 170 kDa was EGF receptor (Figure 5B). This was further verified by immunoprecipitation with anti-phosphotyrosine antibody 4G10, followed by immunoblotting with EGF-receptor antibody (Figure 5C).

To determine whether the Shc family of adaptor proteins were tyrosine phosphorylated after stimulation with EGF, non-infected HeLa cells and HeLa cells, infected with either bacteria expressing the wild-type ExoS or ExoS mutants for 60 min, were compared. HeLa cells were stimulated with EGF for 2 min and cellular lysates were subjected to immunoprecipitation with a

Figure 6 Phosphorylation of PKB/Akt and Erk 1/2 by ExoS induced by inhibition of EGF *in vivo*

Effect of ExoS on PKB/Akt and Erk 1/2 phosphorylation in NIH3T3 (A) and HeLa (B) cells. Phosphorylation of PKB/Akt and Erk 1 and 2 were examined in (lane 1) non-stimulated (–) and (lanes 2–7) EGF-stimulated (+) cells. Cells were infected for 60 min as follows: lanes 1 and 2, uninfected cells; lane 3, mock infection; lane 4, infection with ExoS(E381A,Δ98–232); lane 5, infection with ExoS(E381A); lane 6, infection with ExoS(Δ98–232); lane 7, infection with wild-type ExoS. The whole-cell lysates were subjected to SDS/PAGE followed by immunoblotting with anti-phosphospecific Erk 1/2 (α-phospho-Erk) and PKB/Akt (α-phospho-Akt) antibodies, as indicated. The membranes were stripped and reprobed with anti-Erk and anti-Akt antibodies as indicated. Molecular-mass markers are shown on the right.

polyclonal Shc antibody, followed by immunoblotting with 4G10 anti-phosphotyrosine antibody (Figure 5D). Phosphotyrosine proteins of 46, 52 and 66 kDa were detected in all EGF-stimulated HeLa cell lysates, regardless of whether infected with bacteria expressing wild-type or ExoS mutant proteins, but were absent in non-stimulated lysates (Figure 5D; B. Hallberg and M. L. Henriksson, unpublished work). The membrane was stripped and reprobed with anti-Shc antibody, thus confirming that the phosphorylated bands were Shc proteins (Figure 5E). We then examined whether phosphorylated Shc proteins were still able to interact with the adaptor protein Grb2. As expected the Shc–Grb2 complex was formed and observed in immunoprecipitates, and was independent of ExoS infection (Figure 5F).

Signal transduction components downstream of Ras are inhibited by ExoS

A primary target of activated Ras during EGF stimulation is Raf, which is the first component of a protein kinase cascade that leads to activation of the MAP kinases/Erk 1/2. Another target of activated Ras is phosphatidylinositol 3-kinase, which in turn mediates the activation of PKB/Akt, a serine/threonine kinase. The expected activation of PKB/Akt and Erk1/2 after stimulation by EGF was observed in both cell lines (Figure 6, lanes 1 and 2). However, PKB/Akt and Erk 1/2 phosphorylation was eliminated upon stimulation with EGF after 60 min of infection with *Y. pseudotuberculosis* expressing wild-type ExoS and ExoS(Δ98–232) (Figure 6, compare lanes 1 and 2 with lanes 6 and 7). This effect was not seen with cells infected with ExoS(E381A) and ExoS(Δ98–232, E381A) for 40, 60 or 80 min (Figure 6; M. L. Henriksson and B. Hallberg, unpublished work), sug-

gesting that the ExoS mutant proteins with limited catalytic capacity are unable to block PKB/Akt and Erk 1/2 phosphorylation upon stimulation with EGF.

DISCUSSION

In the present study, we have examined the functional consequences of ADP-ribosylation of the small GTPase protein Ras. We, and others, have previously reported that bacterially translocated ExoS modifies Ras and that ExoS translocation elicits a cytotoxic response correlated with disruption of the actin microfilament structure, decreased viability of the cell and inhibition of proliferation [26,29–33,39]. We have shown that Ras is ADP-ribosylated by ExoS and this modification results in a change in the ratio of GTP/GDP-bound Ras *in vivo*. This ratio is very useful in understanding the importance of Ras in a given signalling process, since the amount of Ras–GTP bound (active) relative to the total amount of Ras–(GTP+GDP) bound in the cell reflects the activity of Ras. HeLa cells, stimulated with EGF at time of infection or uninfected show a 6- to 7-fold increase in Ras–GTP levels. However, HeLa cells infected with bacteria expressing ExoS before stimulation with EGF show a significantly lower degree of Ras activation. In contrast, infection of HeLa cells with the ExoS(E381A) mutant or mock infection did not inhibit Ras–GTP loading in response to EGF.

Further evidence for the inactivation of Ras *in vivo* by ExoS modification can be observed using the RBD of Raf-1. Activated Ras interacts with Raf-1 when Ras is GTP-bound, thereby allowing detection of functionally active cellular Ras. As expected, treatment of HeLa cells with EGF increased the precipitation of Ras by GST–Raf–RBD. However, in HeLa cells after infection with ExoS or ExoS(Δ98–232), both of which contain a complete catalytic domain, the EGF-induced stimulation of Ras–Raf–RBD complex formation is completely blocked by ExoS modification. Interaction between GST–Raf–RBD and Ras is only observed when cells are infected with ExoS mutants with limited catalytic capacity, such as ExoS(E381A) and ExoS(Δ98–232, E381A).

We then investigated the possibility that ExoS is able to affect signalling components upstream of Ras, such as the EGF receptor and the two adaptor proteins, Shc and Grb2. From our results we conclude that neither the wild type ExoS nor the ExoS mutants impair immediate EGF-receptor function, as measured by tyrosine phosphorylation upon ligand-mediated receptor stimulation. In addition, ExoS infection does not appear to affect efficient tyrosine phosphorylation of the adaptor protein, Shc, or complex formation between Grb2 and Shc, upon receptor stimulation with EGF.

Activated Ras plays a role in the control of downstream signalling pathways, including the PKB/Akt and MAP kinase proteins Erk1 and Erk 2. We observed an expected phosphorylation of both PKB/Akt and of Erk 1/2 after stimulation by EGF in HeLa cells [15,17]. However, both PKB/Akt and Erk phosphorylation were abolished upon stimulation with EGF after infection with wild-type ExoS and ExoS(Δ98–232). This effect was not seen with cells infected with ExoS(E381A) or ExoS(Δ98–232, E381A), suggesting that catalytically impaired ExoS mutant proteins are unable to block PKB/Akt and Erk 1/2 activation upon stimulation with EGF. These results suggest that infection of both NIH3T3 and HeLa cells with ExoS blocks the phosphorylation and activation of PKB/Akt and MAP kinases, Erk1 and Erk 2, upon stimulation with EGF, presumably due to down-regulation of Ras activation.

Since ExoS elicits a cytotoxic response correlated with disruption of the actin microfilament structure [26], we investigated

whether RhoA, another small G-protein involved in actin organization in cytoskeleton regulation [2], was modified by ExoS. However, so far, no modification of RhoA after infection with ExoS *in vivo* has been observed, although RhoA was modified by ExoS *in vitro* (M. L. Henriksson and B. Hallberg, unpublished work).

From this series of experiments *in vivo*, we propose that the ExoS protein is able to block activation of Ras upon stimulation with EGF. Our results indicate that this is due directly to the activity of ExoS modification of the Ras protein, as seen *in vitro*. We consider this to be the most likely scenario, as the preferential ADP-ribosylation site in Ras has been identified as arginine-41, which is very close to the effector domain of Ras [38]. Post-translational modification at this site is likely to affect Ras protein function. We, and others, have shown that the glutamic acid residue at position 381 is essential for the ADP-ribosyl-transferase activity of ExoS, and this glutamic acid is thought to constitute part of the active site of this enzyme [26,32]. The precise mechanism by which ExoS is able to affect the bound nucleotide on Ras proteins is presently unclear. However, our results show for the first time that the exchange of GDP for GTP on Ras *in vivo* is inhibited by ExoS, which correlates with the observed ADP-ribosylation of the Ras protein *in vivo* (Figures 2A and 3). Thus it is now clear that ExoS is not only able to modify Ras *in vivo*, but also is capable of affecting Ras activation *in vivo*, which has been suggested by experiments *in vitro* [46]. It is conceivable that this decrease in Ras-GTP loading is due to steric hindrance caused by ADP-ribosylation of Ras itself, although further investigation will be required in order to elucidate the mechanisms involved. Taken together, our results show that Ras is a target *in vivo* for the ExoS of *Ps. aeruginosa*, and that ExoS is able to inhibit Ras activation of downstream signalling pathways.

We thank Ruth Palmer for scientific advice and a critical reading of the manuscript. Thanks to Jonas Ekstrand, Pat Warne and Julian Downward for support. Financial support for this work was from the Elsa and Folke Sahlbergs Fund for Medical Research (B.H.), the Lion's Cancer Research Foundation (B.H.), the Swedish Medical Research Council (R.R. and H.W.-W.), the Swedish Foundation of Strategic Research (H.W.-W.), and the Swedish Cancer Society (B.H.).

REFERENCES

- Aktories, K. (1997) *Trends Microbiol.* **5**, 282–288
- Bos, J. L. (1998) *EMBO J.* **17**, 6776–6782
- Hall, A. (1998) *Science* **279**, 509–514
- Fantl, W. J., Johnson, D. E. and Williams, L. T. (1993) *Annu. Rev. Biochem.* **62**, 453–481
- Exton, J. H. (1998) *J. Biol. Chem.* **273**, 19923
- Hackel, P. O., Zwick, E., Prenzel, N. and Ullrich, A. (1999) *Curr. Opin. Cell. Biol.* **11**, 184–189
- Egan, S. E. and Weinberg, R. A. (1993) *Nature (London)* **365**, 781–783
- Boguski, M. S. and McCormick, F. (1993) *Nature (London)* **366**, 643–654
- Bourne, H. R., Sanders, D. A. and McCormick, F. (1991) *Nature (London)* **349**, 117–127
- Schweins, T. and Wittinghofer, A. (1994) *Curr. Biol.* **4**, 547–550
- Leevers, S. J. and Marshall, C. J. (1992) *EMBO J.* **11**, 569–574
- Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, D. F., Weber, M. J. and Sturgill, T. W. (1991) *EMBO J.* **10**, 885–892
- Vojtek, A. B. and Der, C. J. (1998) *J. Biol. Chem.* **273**, 19925–8
- Hill, C. S. and Treisman, R. (1995) *Cell* **80**, 199–211
- Taylor, S. J. and Shalloway, D. (1996) *Curr. Biol.* **6**, 1621–7
- Marshall, C. J. (1996) *Curr. Opin. Cell Biol.* **8**, 197–204
- Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992) *Biochem. J.* **288**, 351–355
- Downward, J. (1998) *Curr. Opin. Cell Biol.* **10**, 262–267
- Vanhaesebroeck, B., Leever, S. J., Panayotou, G. and Waterfield, M. D. (1997) *Trends Biochem. Sci.* **22**, 267–272
- Leevers, S. J., Vanhaesebroeck, B. and Waterfield, M. D. (1999) *Curr. Opin. Cell Biol.* **11**, 219–225
- Nicas, T. I., Frank, D. W., Stenzel, P., Lile, J. D. and Iglewski, B. H. (1985) *Eur. J. Clin. Microbiol.* **4**, 175–179
- Nicas, T. I. and Iglewski, B. H. (1985) *Can. J. Microbiol.* **31**, 387–392
- Kudoh, I., Wiener-Kronish, J. P., Hashimoto, S., Pittet, J. F. and Frank, D. (1994) *Am. J. Physiol.* **267**, L551–L556
- Rosqvist, R., Hakansson, S., Forsberg, A. and Wolf-Watz, H. (1995) *EMBO J.* **14**, 4187–4195
- Yahr, T. L., Goranson, J. and Frank, D. W. (1996) *Mol. Microbiol.* **22**, 991–1003
- Yahr, T. L., Mende-Mueller, L. M., Fries, M. B. and Frank, D. W. (1997) *J. Bacteriol.* **179**, 7165–7168
- Frithz-Lindsten, E., Du, Y., Rosqvist, R. and Forsberg, A. (1997) *Mol. Microbiol.* **25**, 1125–1139
- Krueger, K. M. and Barbieri, J. T. (1995) *Clin. Microbiol. Rev.* **8**, 34–47
- Iglewski, B. H., Sadoff, J., Bjorn, M. J. and Maxwell, E. S. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3211–3215
- Rosqvist, R., Magnusson, K. E. and Wolf-Watz, H. (1994) *EMBO J.* **13**, 964–972
- Pederson, K. J., Vallis, A. J., Aktories, K., Frank, D. W. and Barbieri, J. T. (1999) *Mol. Microbiol.* **32**, 393–401
- Pederson, K. J. and Barbieri, J. T. (1998) *Mol. Microbiol.* **30**, 751–759
- Liu, S., Kulich, S. M. and Barbieri, J. T. (1996) *Biochemistry* **35**, 2754–2758
- Knight, D. A., Finck-Barbancon, V., Kulich, S. M. and Barbieri, J. T. (1995) *Infect. Immun.* **63**, 3182–3186
- Coburn, J., Kane, A. V., Feig, L. and Gill, D. M. (1991) *J. Biol. Chem.* **266**, 6438–6446
- Fu, H., Coburn, J. and Collier, R. J. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2320–2324
- Coburn, J., Wyatt, R. T., Iglewski, B. H. and Gill, D. M. (1989) *J. Biol. Chem.* **264**, 9004–9008
- Coburn, J., Dillon, S. T., Iglewski, B. H. and Gill, D. M. (1989) *Infect. Immun.* **57**, 996–998
- Ganesan, A. K., Frank, D. W., Misra, R. P., Schmidt, G. and Barbieri, J. T. (1998) *J. Biol. Chem.* **273**, 7332–7337
- McGuffie, E. M., Frank, D. W., Vincent, T. S. and Olson, J. C. (1998) *Infect. Immun.* **66**, 2607–2613
- Persson, C., Nordfelth, R., Holmstrom, A., Hakansson, S., Rosqvist, R. and Wolf-Watz, H. (1995) *Mol. Microbiol.* **18**, 135–150
- Petersson, J., Nordfelth, R., Dubinina, E., Bergman, T., Gustafsson, M., Magnusson, K. E. and Wolf-Watz, H. (1996) *Science* **273**, 1231–1233
- Basu, T., Warne, P. H. and Downward, J. (1994) *Oncogene* **9**, 3483–3491
- Hallberg, B., Rayter, S. I. and Downward, J. (1994) *J. Biol. Chem.* **269**, 3913–3916
- Downward, J., Graves, J. D., Warne, P. H., Rayter, S. and Cantrell, D. A. (1990) *Nature (London)* **346**, 719–723
- Nakafuku, M., Satoh, T. and Kaziro, Y. (1992) *J. Biol. Chem.* **267**, 19448–19454
- Ganesan, A. K., Vincent, T. S., Olson, J. C. and Barbieri, J. T. (1999) *J. Biol. Chem.* **274**, 21823–21829

Received 22 October 1999/4 January 2000; accepted 26 January 2000