Expression of *Pseudomonas aeruginosa* Toxin ExoS Effectively Induces Apoptosis in Host Cells[▽]

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Received 20 April 2006/Returned for modification 31 May 2006/Accepted 4 September 2006

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that primarily infects immunocompromised individuals and patients with cystic fibrosis. Invasive strains of *P. aeruginosa* are known to induce apoptosis at a high frequency in HeLa cells and in many other cell lines, a process that is dependent on the ADP-ribosylation (ADPRT) activity of a type III secreted protein ExoS. In our previous report, it was proposed that *P. aeruginosa* secreting ExoS, upon infection, shuts down host cell survival signal pathways by inhibiting ERK1/2 and p38 activation, and it activates proapoptotic pathways through activation of JNK1/2, leading ultimately to cytochrome *c* release and activation of caspases. In this study, we demonstrate that the expression of ExoS in HeLa cells by eukaryotic expression vector effectively caused apoptosis in an ADPRT activity-dependent manner, indicating that ExoS alone is sufficient to trigger apoptotic death of host cells independent of any other bacterial factors. By expressing an EGFP-ExoS fusion protein, we were able to directly correlate the death of HeLa cells with the presence of intracellular ExoS and further proved the dependence of this process on both JNK activation and mitochondrial proapoptotic event. The cellular pathway responsible for the ExoS-induced cytotoxicity appears to be well conserved, since the expression of the ADPRT-competent ExoS also induced rapid cell death in the *Drosophila melanogaster* S2 cell lines. The presented study not only highlights the ability of ExoS ADPRT to modulate host cell signaling, eventually leading to apoptosis, but also establishes ExoS as a valuable tool, in principle, for the elucidation of apoptosis mechanisms.

Pseudomonas aeruginosa, an opportunistic bacterial pathogen, causes devastating human infections in patients with cystic fibrosis, severe burns, or immunosuppression (31, 52, 74). To successfully colonize and maintain its infectious cycle, *P. aeruginosa* orchestrates the production of a large arsenal of virulence factors in a highly regulated manner (35, 84). Some of the virulence factors are produced and injected directly into the host cell by using the cell contact-mediated type III secretion system (TTSS), which has attracted significant attention in recent years (18, 28, 44). To date, four TTSS effector molecules have been described in *P. aeruginosa*. Exoenzyme U (ExoU) is a necrotizing toxin with phospholipase activity (39, 81), and ExoY is an adenylate cyclase (92). The other two known effectors are ExoS and ExoT, highly homologous to each other, having a carboxy-terminal ADP-ribosyltransferase (ADPRT) domain and an amino-terminal GTPase-activating (GAP) domain (32, 53, 54, 76, 85).

The GAP activity of ExoS and ExoT targets small Rho-like GTPases, such as Rho, Rac, and Cdc42 (1, 53, 54), and has been linked to cytoskeletal rearrangements and cell rounding in vitro (32, 53). Although ExoS and ExoT share 75% amino acid identity, their ADPRT domains appear to have distinctive groups of target proteins in host cells (1, 86). The ADPRT activity of ExoS has been shown to modify Ras and several Ras-like host proteins in vivo, including RalA, Rabs, and Rac1 (5, 15, 25), which is linked to cytotoxicity toward eukaryotic

cells (70); it also has non-G-protein substrates, the Ezrin/Radixin/Moesin (ERM) family of proteins (62), demonstrating another mechanism by which ExoS can modulate cytoskeleton dynamics. Glutamic acid at position 381 (E381) functions as a catalytic residue for the ExoS ADPRT domain (76), the activation of which requires a 14-3-3 family protein, termed FAS (for factor for activating ExoS), from eukaryotic host (27).

In addition, we reported that the ADPRT activity of the ExoS was required for triggering rapid apoptosis in various host cells upon infection by invasive strains of *P. aeruginosa* (50). In that study, the apoptotic death in infected cells was determined by several criteria, including (i) visual changes in cell morphology, (ii) the induction of chromatin condensation and nuclear marginalization, (iii) the presence of a high percentage of cells with subG1 DNA content, and (iv) the activation of caspase-3 activity. Subsequently, evidence was provided to suggest that, in infected host cells, *P. aeruginosa* producing ExoS not only triggers a proapoptotic pathway through JNK-mediated cytochrome *c* release from mitochondria but also sensitizes the host cell to proapoptotic signals by inhibiting antiapoptotic pathway(s) controlled by ERK1/2 and possibly also by p38 (48).

There are three major regulatory pathways known to regulate caspase-based death programs: the mitochondrial pathway, which involves Bcl-2 family proteins and *ced-4*/Apaf-1 (43, 61), the *reaper*-family/inhibitor of apoptosis protein (IAP) pathway (64, 99), and the death receptor pathway (68). Components of all three pathways have been documented in both mammals and insects, and perturbation of each of these pathways has been implicated in a variety of human diseases (40).

In the present study, by using multiple approaches to express ExoS with eukaryotic expression vectors, we demonstrate that

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[▽] Published ahead of print on 11 September 2006.

TABLE 1. List of bacterial strains and plasmids

Strain or plasmid	Description ^a	Source or reference
Strains		
<i>E. coli</i> DH5 α	F ⁻ ϕ 80 <i>dlacZ</i> Δ M15 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 relA1</i> Δ (<i>lacZYA-argF</i>) <i>U169 gyrA96 deoR</i>	37
<i>P. aeruginosa</i> PAK	Laboratory strain (invasive)	D. Bradley ^b
PAK <i>exoS::</i> Ω / <i>exoT::</i> Gem	<i>exoS</i> <i>exoT</i> double-mutant derivative of PAK	50
Plasmids		
pCR2.1-TOPO	PCR cloning vector; Ap ^r Km ^r	Invitrogen
pcDNA4	Constitutive mammalian expression vector with CMV promoter; Ap ^r	Invitrogen
pJJ0040	<i>exoS</i> of PAK cloned into pcDNA4; Ap ^r	This study
pJJ0042	<i>exoS</i> of pJJ0040 mutated to <i>exoS</i> (R146K); Ap ^r	This study
pJJ0043	<i>exoS</i> of pJJ0040 mutated to <i>exoS</i> (E381A); Ap ^r	This study
pJJ0044	<i>exoS</i> of pJJ0040 mutated to <i>exoS</i> (R146K/E381A); Ap ^r	This study
pUCP18	Broad-host-range cloning vector, IncP; Ap ^r	82
pHW0015	<i>exoS</i> derived from PAK in pUCP18; Ap ^r	50
pExoSRK18	<i>exoS</i> (R146K) in pUCP18; Ap ^r	This study
pExoSEA18	<i>exoS</i> (E381A) in pUCP18; Ap ^r	This study
pExoSRKEA18	<i>exoS</i> (R146K/E381A) in pUCP18; Ap ^r	This study
pEGFP-C1	Constitutive mammalian expression vector containing <i>egfp</i> (CMV promoter); Km ^r	BD Clontech
pJJ0322	<i>exoS</i> in pEGFP-C1; Km ^r	This study
pJJ0323	<i>exoS</i> (E381A) in pEGFP-C1; Km ^r	This study
pJJ0324	<i>exoS</i> (R146K/E381A) in pEGFP-C1; Km ^r	This study
pJJ0325	<i>exoS</i> (R146K) in pEGFP-C1; Km ^r	This study
pUF5	Reporter construct pGFP	A. Lewin
pcDNA4	Constitutive mammalian expression vector with CMV promoter; Ap ^r	Invitrogen
pie3	<i>Drosophila</i> expression vector; Ap ^r	Invitrogen
pie_exoS	<i>exoS</i> in pie3; Ap ^r	This study
pie_exoSRK	<i>exoS</i> (R146K) in pie3; Ap ^r	This study
pie_exoSEA	<i>exoS</i> (E381A) in pie3; Ap ^r	This study
pie_ExoSdouble	<i>exoS</i> (R146K/E381A) in pie3; Ap ^r	This study
pie_reaper	<i>reaper</i> in pie3; Ap ^r	49
pie_Diap1	<i>Diap1</i> in pie3; Ap ^r	98

^a Antibiotic resistance markers: Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

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ExoS alone, precisely the ADPRT of ExoS, is sufficient to trigger apoptosis in cultured host cells representing human and drosophila, independent of any other signals from the bacterial cell. Also importantly, the apoptosis induced by exogenously expressed ExoS was proven dependent on both JNK activation and mitochondrial proapoptotic event. Therefore, by extending our previous findings on *P. aeruginosa*-induced apoptosis in distinctive approaches, the presented data set up a foundation for us to take the advantage of a novel role ExoS may play in studying apoptosis mechanisms in eukaryotic cells.

MATERIALS AND METHODS

Materials and cell lines. Eukaryotic cell expression vectors, pcDNA4 and pEGFP-C1, were obtained from Invitrogen (Carlsbad, CA) and BD Bioscience-Clontech (Palo Alto, CA), respectively. Reporter construct pGFP (pUF5) was kindly provided by Alfred Lewin (University of Florida College of Medicine). Transfection reagents Lipofectamine Plus and Lipofectamine 2000 were purchased from Invitrogen. Anti-green fluorescent protein (anti-GFP; 1:1,000) and anti-actin (1:500) antibodies, used at the indicated dilutions, were obtained from Santa Cruz Biotech (Santa Cruz, CA). Anti-ExoS and anti-ExoT antibodies were generated from rabbits by Lampire Biological Laboratories, Inc. (Pipersville, PA). Peroxidase-conjugated goat anti-rabbit secondary antibodies and peroxidase-conjugated goat anti-mouse antibodies were obtained from Santa Cruz Biotech and Amersham Pharmacia Biotech (Piscataway, NJ), respectively. SP600125 (SP), staurosporine (STS), cycloheximide (CHX), caspase-3 inhibitor DEVD-CHO, and pan-caspase inhibitor Boc-D-FMK were obtained from CalBiochem (San Diego, CA).

HeLa cell lines stably transfected with Bcl-x_L or Neo vector alone were generously provided by Xiaodong Wang (University of Texas Southwestern Medical Center, Dallas) and grown as suggested previously (69). Other HeLa cell cultures were maintained or treated as previously described (48).

Bacterial strains and plasmids. Strains and plasmids used in the present study are listed in Table 1. Bacterial cultures were grown in Luria-Bertani broth at 37°C. To construct pcNDA4-*exoS*, *exoS* was cloned by PCR, using PAK chromosomal DNA as a template with the forward primer 5'-CAG GAG AAG GTA CCA TCA TGG ATA TTC AAT CGC TTC AG-3' (underlining indicates the mutated nucleotides) and the reverse primer 5'-CGT TTC GTC GCC TGG ACC TAC CTC GAC AAG AAG CA-3'. The forward primer contained the KpnI restriction site (GGTACC) upstream of the *exoS* initiation codon and Kozak translation initiation sequence (i.e., G/ANNATGG). The PCR product was cloned into pCR2.1-TOPO vector (Invitrogen, Inc.), the resulting plasmid (pCR 2.1 TOPO-*exoS*) was then digested with KpnI/EcoRI, and the *exoS*-containing fragment was further ligated into the same site of pcDNA4 to generate pcNDA4-*exoS* (pJJ0040), where *exoS* is under the control of cytomegalovirus (CMV) promoter. The pcNDA4-*exoS* was sequenced to confirm the correct coding sequence as well as the fusion junction.

Site-directed mutagenesis. Additional mutations of the *exoS* in pJJ0040, such as *exoS*(E381A) (ADPRT null), *exoS*R146K (GAP null), and *exoS*(R146K)/*exoS*(E381A) (ADPRT and GAP null double mutant), were generated by site-specific mutagenesis, resulting in pJJ0043, pJJ0042, and pJJ0044, respectively. The oligonucleotides used to generate the mutations were as follows: for *exoS*(R146K), 5'-GAG ATG GGG CCC TGA AAT CGC TGA GCA CC-3', in which the Arg codon CGT was replaced with the Lys codon AAA while creating a new ApaI site; and for *exoS*(E381A), 5'-GAA TGA AAA AGC AAT ATT GTA TAA CAA AGA G-3', where the Glu codon GAG was replaced with the Ala codon GCA while creating a new SspI site. The creation of new restriction sites allowed quick identification of mutant plasmids in initial screening. Base

changes in the pcDNA4 constructs were verified by DNA sequencing. Subsequently, clones of the *exoS(E381A)* and *exoS(R146K)* in pUCP18 were generated from pJJ0043 and pJJ0042, respectively, by replacing the corresponding mutant fragments with that of pHW0015 (*exoS* clone in pUCP18 or pUCP18*exoS*). In this procedure, a ClaI-XbaI fragment was used to generate a pUCP18 containing *exoS(E381A)* (pExoSEA18), whereas XmaI was used for *exoS(R146K)* (pExoSRK18).

Transfection of HeLa cells. Cells were plated on six-well plates at 1.5×10^5 cells/well in Dulbecco modified Eagle medium (DMEM) plus 10% fetal calf serum (FCS), 100 U of penicillin G sodium/ml, 100 μ g of streptomycin sulfate/ml, and 2 mM L-glutamine. After 24 h, cells were transfected with the indicated constructs or vector control (2 μ g/well for transfection or 6 μ g/well in total for cotransfection) by using Lipofectamine 2000 reagent (Invitrogen) or Lipofectamine-Plus reagent according to the manufacturer's instructions. To select for stable transfectants, growth medium was replaced at 48 h posttransfection and then weekly for 3 weeks with fresh medium containing 600 μ g of G418 (Life Technologies, Inc.)/ml.

Western blot analysis of ExoS and EGFP-ExoS. At various times postinfection, HeLa cells were collected and suspended in protein loading buffer (62.5 mM Tris-HCl [pH 6.8], 2% [wt/vol] sodium dodecyl sulfate [SDS], 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue). Equal amounts of total protein samples were subjected to SDS–12% polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, the proteins were transferred to PVDF-Plus membranes (Osmonics, Inc., Minnetonka, MN) at 50 mA for 60 min using a semidry protein transfer system (Bio-Rad, Hercules, CA). Blots were blocked with 5% (wt/vol) dry milk in 1 $\times$ TBS buffer (50 mM Tris-HCl, 150 mM NaCl [pH 7.4]) containing 0.5% (vol/vol) Tween 20 for 60 min and then probed with the appropriate antibodies for 1 h at room temperature or overnight at 4°C, according to suppliers' recommendations. After being washed with 1 $\times$ TBS–0.1% Tween 20, the membranes were incubated with peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies for 60 min. Specific signals were developed by using the ECL-Plus system (Amersham). To ensure equal loading and even transfer of proteins, protein bands on the membrane were visualized by staining with Ponceau S (0.1% Ponceau S in 3% trichloroacetic acid [wt/vol]) and further reprobed with anti-actin antibody after treatment with stripping buffer (62.5 mM Tris-HCl [pH 6.7], 100 mM mercaptoethanol, and 2% [vol/vol] SDS) for 20 min at 60°C. The bands were quantified by using the densitometry program LabWorks (UVP, Inc., Upland, CA). All measurements of protein levels were normalized against the β -actin signal. Each Western blot experiment was conducted with two separate membranes in parallel to ensure reproducibility.

Infection of HeLa cells by *P. aeruginosa*. HeLa cell monolayers were plated from suspension culture 1 day prior to infection in DMEM supplemented with 5% FCS (DMEM–5% FCS). HeLa cell monolayers ($\sim 5 \times 10^5$ cells per well; >80% confluence) were washed with phosphate-buffered saline (PBS), mixed with bacteria (10^7 CFU/ml in DMEM; multiplicity of infection of 20), and incubated for 2 h at 37°C in a 5% CO₂ incubator. The cells were washed with PBS to remove the nonadhering bacteria. Fresh medium, DMEM–5% FCS supplemented with 400 μ g of gentamicin or amikacin/ml, was added, and the cells were incubated for an additional 3 to 24 h. As positive controls for the apoptosis, HeLa cells were incubated with 10 ng of tumor necrosis factor (TNF)/ml and 20 μ g of CHX/ml or with 2 μ M STS.

Caspase-3 activation analysis. Caspase-3 activity was measured by using the caspase-3 cellular activity assay kit plus (BioMol). HeLa cells (3×10^7) infected with *P. aeruginosa* were washed and harvested by scraping and centrifugation (1,000 $\times$ g, 10 min) at various times postinfection. Cells were lysed with caspase-3 assay lysis buffer (BioMol) containing 0.1% Tween 20, and the cell lysates were centrifuged at 10,000 $\times$ g for 10 min. Dilutions of the cell lysates in a 96-well plate were incubated in triplicate with caspase-3 substrate DEVD-pNA, or the substrate plus caspase-3 inhibitor DEVD-CHO. Changes in the optical density at 405 nm were monitored for 2 h at 10-min intervals. Protein concentrations were determined by using the protein assay system from Bio-Rad. The specific activity is reported as picomoles of substrate cleaved/minute per microgram of protein.

Alternatively, caspase-3 activation in EGFP-ExoS-transfected HeLa cells was assayed by fluorescence-activated cell sorting (FACS) analysis. HeLa cells seeded in a six-well plate were cultivated for 1 day before transfection with EGFP-ExoS fusion constructs. The HeLa cells were collected 24 h later, and the caspase-3 activity was measured using Red-DEVD-FMK (Oncogene Research Products, San Diego, CA), which is a cell-permeable, nontoxic caspase-3 inhibitor conjugated to a sulforhodamine fluorescent marker. It binds irreversibly and selectively to activated caspase-3 at an early stage of apoptosis, and the red fluorescence label allows for the direct detection of activated caspase-3 in apoptotic cells by flow cytometry (excitation max, $\sim$ 540 nm; emission max, $\sim$ 570 nm).

Flow cytometry analysis was performed using FL-2 channel on FACSsort cytometer (BD Biosciences, San Jose, CA). Dot density analysis of each sample was represented as the FL-2 intensity of a single cell (x axis) and the forward light scatter of cells (y axis). As cells undergo apoptosis, their size gradually decreases, resulting in decreased forward light scatter. At the same time, leakage of cytosolic proteins in general results from compromised membrane integrity at the late stage of apoptosis. The index for a gating population with activated caspase-3 in this assay was determined by the analysis pattern for HeLa cells treated with 2 μ M STS for 4 h, which served as a positive control. In all cases, 10,000 cells were analyzed by flow cytometry. The figure generation and data analysis were done by using FCS Express 2 program (De Novo Software, Ontario, Canada).

Hoechst staining of condensed chromatin. Transfected cells were recovered by trypsinization of the cell monolayer. Cells were washed once with PBS and stained with Hoechst 33258 (Molecular Probes, Inc., Eugene, OR) at 1 mg/ml for 10 min in the dark. Chromatin condensation was examined under the fluorescence microscope by using a DAPI (4',6'-diamidino-2-phenylindole) filter after stained cells were mounted onto slides using mounting medium (Vectashield Hard Set; Vector Laboratories, Inc., Burlingame, CA).

Cell death assay in *Drosophila* S2 cell line. The coding regions for *exoS*, *exoS(E381A)*, *exoS(R146K)*, and *exoS(R146K/E381A)* were cut out from the respective pcDNA4 constructs and subcloned into the Pic3 vector (Invitrogen), resulting in *pie_exoS*, *pie_exoSEA*, *pie_exoSRK*, and *pie_exoSRKEA*, respectively. A cell death assay of the *Drosophila* S2 cell line was performed as described previously (98). Briefly, for each test, 1.0 μ g of DNA was distributed into 2 wells in a 24-well plate. This included 0.2 μ g of pIE-lacZ and 0.8 μ g of the test DNA sample or a combination of samples in the pIE vector. Intact pIE vector was used as the vector control. At 20 h posttransfection, cells were fixed and stained with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside)–IPTG (isopropyl- β -D-thiogalactopyranoside). Blue cells were counted to calculate the percentage of cell survival. All experiments reported here were repeated at least three times.

Statistics. All data represent at least three independent experiments and are expressed as the means $\pm$ the standard deviations (SD) unless otherwise indicated. Differences between groups were compared by using the Student *t* test, for pair comparisons, to generate *P* values. Unless otherwise indicated, *P* values are indicated in all of figures and tables as follows: *, *P* < 0.05; **, *P* < 0.01; and ***, *P* < 0.001.

RESULTS

Transiently expressed ExoS induces apoptosis in HeLa cells. In a previous study, we demonstrated that ExoS is the effector molecule required for the *P. aeruginosa*-induced rapid and high-frequency apoptosis in HeLa and other cell lines (50). Analysis of mutant forms of the ExoS further suggested that its ADPRT activity is essential for eliciting apoptosis (50). We intended to further dissect signaling pathways involving ExoS-mediated apoptosis. *P. aeruginosa* possesses a large array of virulence factors that can cause host cell toxicity to certain extent (2, 7, 8, 34, 47, 50, 60, 67, 75, 88, 93, 95); at the same time, it has not been demonstrated conclusively whether ExoS-dependent apoptosis induction coincides with necrosis-like cell death resulting from the diverse effects on cellular components by ExoS or whether apoptosis is secondary to that event. It is therefore important to determine whether the ExoS protein alone is sufficient to induce apoptosis in host cells independent of other bacterial virulence components. To this end, attempts were made to establish a stably transfected HeLa cell line with inducible expression of the *exoS* using eukaryotic expression vector T-REx expression system (Invitrogen, Inc.). In this system, the transcription of the gene of interest is blocked in the absence of an inducer, such as tetracycline or doxycycline, but is permitted or activated after the inducer is added into culture medium (33, 94). However, the desired stable cell line carrying *exoS* could not be generated after repetitive rounds of selection. Analysis of a few resulting colonies showed the loss of the

exoS gene (data not shown). Since we were able to generate stable cell lines carrying *exoS*(R146K/E381A) (GAP and ADPRT null) or vector control, the failure to generate an *exoS*-expressing cell line is presumably due to the basal level expression of the ADPRT capable *exoS*, which caused effective cell death.

Therefore, a transient-expression system was adopted. HeLa cells were transiently cotransfected with wild-type or mutant forms of the *exoS*, driven by the CMV promoter, together with a reporter plasmid expressing GFP. GFP is unique in that its fluorophore forms spontaneously without added cofactors. As a result, the emitted fluorescence intensity provides a direct readout of GFP expression (11), which can be measured at the single-cell level without any processing steps.

To ensure the *exoS* presence in HeLa cells expressing reporter GFP, a fivefold excess of *exoS*-expressing plasmid (pExoS) or vector control plasmid (pcDNA4) was used over the reporter plasmid pGFP (pUF5) in all cotransfections. At 24 h after the cotransfection of vector pcDNA4 and pGFP, a significant fraction of the population was GFP positive (data not shown), and quantitative analysis indicated cotransfection efficiencies between 15 and 30%. In contrast, cotransfection with the pExoS in the equivalent population resulted in fewer and lower-intensity GFP-positive cells, suggesting that wild-type ExoS protein caused significant cytotoxicity (data not shown). Moreover, the application of cell-permeable caspase-3

FIG. 1. Caspase-3-dependent cytotoxicity caused by transfection of the *exoS* expression plasmid. (A) Disappearance of GFP signal after cotransfection. HeLa cells were cotransfected with pGFP reporter plasmid and the *exoS* expression plasmids as indicated, with (■) or without (□) the presence of 1 μ M DEVD-CHO, a cell-permeable caspase-3 inhibitor. HeLa cells were observed under a fluorescence microscope using GFP fluorescence at 24 h posttransfection, and GFP-positive cells were quantified from five random views. Plasmids used for cotransfection with pGFP are indicated as follows: pcDNA4, vector control; pexoS, wild-type *exoS* in pcDNA4(pJJ0040); pexoSEA, *exoS* (E381A) in pcDNA4(pJJ0043); pexoSRK, *exoS*(R146K) in pcDNA4(pJJ0042); and pexoSRKEA, *exoS*(R146K/E381A) in pcDNA4(pJJ0044). As a solvent control, HeLa cells were treated with dimethyl sulfoxide (DEVD-CHO, 0 M). The data are means $\pm$ the SD of the counts of GFP-positive cells from three replicates. *P* values were calculated by comparing DEVD-CHO-treated (1 μ M) and untreated (0 M) groups (***, *P* < 0.001). (B) Nuclear condensation caused by transfection with pcDNA4-*exoS*. HeLa cells were transfected with pexoS, wild-type *exoS* in pcDNA4, or pcDNA4 alone. HeLa cells were collected at 36 h posttransfection, stained with Hoechst dye, and subjected to fluorescence microscopy. Five fields were randomly sampled from each experimental population, and all of the cells stained with Hoechst dye in each field were counted up to 500 in total. The total number of apoptotic cells with condensed or fragmented nuclei was determined in the five sampled regions and was expressed as follows: percentage of apoptosis per sample = (number of apoptotic cells/total number of cells) $\times$ 100%. During and after transfection, HeLa cells were treated with the indicated amount of DEVD-CHO, a cell-permeable specific caspase-3 inhibitor. The data are means $\pm$ the SD of the percentages from three replicates. Significant differences between certain DEVD-CHO treated and untreated groups are indicated (**, *P* < 0.01). (C) Apoptosis after transfection with the indicated *exoS* expression constructs. In each experiment, the apoptotic nucleus percent ratio was calculated by comparing the calculated percentage of apoptosis to that of the pcDNA4. During and after transfection, HeLa cells were treated without (control) or with indicated amounts of DEVD-CHO or VAD-fmk, a cell-permeable specific caspase-3 or a pan-caspase inhibitor, respectively. TNF/CHX, treatment with TNF- α and CHX was used as a positive control for apoptosis induction. The data are means $\pm$ the SD of the ratios from three replicates. Significant differences between certain DEVD-CHO- or VAD-fmk-treated and untreated control groups are indicated (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).

inhibitor DEVD-CHO (Fig. 1) or pan-caspase inhibitor Z-VAD-FMK (data not shown) resulted in significantly increased number of GFP-positive cells after the cotransfection, suggesting that the cytotoxicity elicited by the pExoS was dependent on the activation of caspases.

To quantify apoptosis events, a Hoechst staining assay was

FIG. 2. GAP activity of ExoS is not essential for apoptosis induction by PAK. HeLa cells were infected with the indicated PAK derivative strains (multiplicity of infection of 20) or left uninfected (CTRL). At 5 h postinfection, cells were collected, lysed, and subjected to caspase-3 assay. The *exoS* and *exoT* double mutant PAK (ST) was complemented with vector (ST/V) or with *exoS* (ST/S), *exoS*(E381A) [ST/s(EA)], *exoS*(R146K) [ST/s(RK)], or *exoS*(R146K/E381A) [ST/s(RKEA)] on the same vector. The data are means $\pm$ the SD of the ratios from four replicates. Significant differences between ST/V (V) and other PAK-derived strains are indicated (**, $P < 0.01$; ***, $P < 0.001$).

conducted to assess nuclear condensation and chromatin fragmentation (50). HeLa cells were transiently transfected with vector or pExoS before being subjected to the analysis. About 10% of cells had apoptotic nucleus changes resulting from the expression of vector control (Fig. 1B), which was considered the basal level of apoptosis caused by the transfection procedure. Remarkably, expression of the *exoS* resulted in more than 35% of the total cells with condensed nuclei, which was significantly reduced by the treatment of HeLa cells with the caspase-3 inhibitor DEVD-CHO in a dose-dependent manner (Fig. 1B), echoing the GFP signal rescue effect.

ADPRT activity of ExoS is sufficient, whereas GAP activity is not required, for apoptosis induction. In contrast to observations with *exoS* (pJJ0040), cotransfection of the ADPRT-null mutant *exoS*(E381A) (pJJ0043) and pGFP showed frequencies of GFP-positive cells similar to those for the expressing vector control (data not shown). Furthermore, the GAP-null mutant *exoS*(R146K) (pJJ0042) caused cytotoxicity as dramatic as the wild-type *exoS* strain did (data not shown). Therefore, the specificity of cytotoxicity correlated with the ADPRT but not the GAP activities. Importantly, all of the GFP-positive cells cotransfected with the *exoS*(E381A) showed rounding morphology (data not shown), a phenomenon presumably caused by the GAP activity of the ExoS through its effect on small Rho GTPases (73). In addition, caspase inhibitors effectively reversed the cytotoxicity caused by the expression of *exoS* (pJJ0040) and *exoS*(R146K) (pJJ0042) (Fig. 1B and C), suggesting the apoptotic nature of the cytotoxicity. These observations were reproduced in different cell lines, such as 293T cells (data not shown), suggesting that this effect is not restricted to the HeLa cell line, an observation consistent with our previous findings in *P. aeruginosa* infection studies (50).

The dispensable role of ExoS GAP activity for apoptosis

FIG. 3. Expression of EGFP fusion proteins in HeLa cell after transient transfection. Wild type and *exoS*, *exoS*(E381A), and *exoS*(R146K/E381A) mutants were cloned into pEGFP-C1, with the resulting fusion constructs labeled as follows: S, wild-type ExoS; EA, ExoS E381A; and RKEA, ExoS R146K E381A double mutant. At 12 h posttransfection, the total cellular proteins were collected. The expression of EGFP or fusion proteins was determined by Western blotting with antibodies to ExoS or GFP. Arrows point to the full-length fusion protein band; the arrowhead indicates the EGFP band. Equal protein loading was controlled by Ponceau S staining of membranes and Western blotting for β -actin.

induction was further investigated by using a previously described bacterial infection model (50). Strain PAK*exoST*, defective of *exoS* and *exoT*, was complemented by the GAP-defective *exoS*(R146K) or other *exoS* clones, namely, wild-type *exoS*, *exoS*(E381A), or the *exoS*(R146K/E381A) double mutant (ADPRT and GAP null). As a negative control, vector pUCP18 was used. There was no noticeable difference in bacterial abilities to adhere to host cells among these strains. After infection of HeLa cells the PAK*exoST*/*exoSR146K* strain, unlike the vector control strain PAK*exoST*/pUCP18, caused a significant level of caspase-3 activation (Fig. 2), as well as nucleus condensation (data not shown). It appeared to be slightly less effective than complementation with wild-type *exoS*, suggesting a minor role for the ExoS GAP activity in sensitizing HeLa cells to apoptosis induction. Finally, as expected, the strain complemented with *exoS*(R146K/E381A) was as "silent" as the vector control strain. In summary, transient expression of *exoS* is sufficient to elicit apoptosis, and this is dependent on ADPRT but not on GAP activity of the ExoS.

Transiently expressed EGFP-ExoS fusion protein effectively induces apoptosis in HeLa cells. To better identify the HeLa cells expressing ExoS and to assess coincident apoptosis, wild-type *exoS* and its mutant derivatives were cloned into a pEGFP-C1 vector to produce the fusion proteins of EGFP-ExoS (pJJ0322), EGFP-ExoS E381A (pJJ0323), and EGFP-ExoS R146K E381A (pJJ0324). With a 15-amino-acid polypeptide as a spacer/linker, the chimeric proteins are 707 amino acids in length. The fusion constructs, as well as the pEGFP control vector, were transfected into HeLa cells, and the expression of the fusion proteins in the resulting HeLa cells was examined by Western blotting. At 12 h posttransfection, the 75-kDa full-length fusion proteins were readily recognizable by an antibody directed against either ExoS or GFP (Fig. 3). The amount of EGFP-ExoS was significantly less than that of the other two mutant fusion proteins, namely, EGFP-SE381A

FIG. 4. (A) Transient expression of EGFP-ExoS in HeLa cells. HeLa cells were transfected with EGFP fusion protein constructs as indicated. HeLa cells were observed under a microscope using GFP fluorescence (A to D) or a bright-field (BF) filter (E to H) at 16 h posttransfection. A and E, EGFP vector control; B and F, EGFP-S, wild-type *exoS* in fusion construct; C and G, EGFP-EA, *exoS*(E381A) in fusion construct; D and H, EGFP-RKEA, *exoS*(R146K) in fusion construct. In each pair of images, the arrows refer to the same cells with detectable GFP. (B) FACS analysis of GFP signals in HeLa cells transiently transfected with fusion constructs. HeLa cells were collected at 24 h posttransfection and subjected to flow cytometry analysis using FL-1 channel on a FACS Sort cytometer. Each color-coded line on the histogram represents a collection of cells that were transfected with an indicated construct, with the GFP intensity of each cell on the *x* axis and counts of the cells with the same intensity on the *y* axis. A threshold intensity for a GFP-positive cell (GFP+ in the figure) was set based on a vector control, and the percentages for GFP-positive cells, of the total, are presented on the right. CTRL means no plasmid DNA was added for transfection. The results are from a single experiment that is representative of three replicated experiments. (C) Cytosolic localization of fusion proteins in HeLa cells after transient transfection with fusion protein expression plasmids. HeLa cells were transfected with EGFP fusion protein constructs as indicated. The resulting HeLa cells were fixed for 16 h after transfection and then stained with DAPI and observed under a phase-contrast DMIRB inverted fluorescence microscope (Leica, Germany) using a GFP fluorescence (a and b) or UV (DAPI, c and d) filter; merged images of GFP and DAPI are also shown (e and f). Panels a, c, and e show EGFP-EA [*exoS*(E381A)] and panels b, c, and f show EGFP-RKEA [*exoS*(R146K/E381A)] in fusion constructs.

(EA) and ExoSR146KE381A (RKEA). Also notably, the protein levels of EGFP-ExoS started to decrease at 12 h posttransfection, whereas those of the two mutant proteins kept rising steadily until 36 h posttransfection (data not shown).

Next, we evaluated cell morphology and GFP fluorescence in HeLa cells transfected with the chimera constructs. Upon transient expression of EGFP-ExoS, very few GFP-positive cells were detectable throughout the 48 h of obser-

vation (Fig. 4A and data not shown). Within the same time frame, a large fraction of the cells gradually developed apoptotic morphology, such as membrane blebbing and nuclear condensation. In contrast, HeLa cells transfected with the other three constructs (EGFP, EGFP-ExoSE381A, or EGFP-ExoSR146KE381A) all resulted in high proportions of GFP-positive population, with significantly higher GFP intensity (Fig. 4A). These results strongly indicated that

FIG. 4—Continued.

expression of the EGFP-ExoS caused substantial cytotoxicity in HeLa cells. These results were also consistent with the evaluation of the GFP signal in HeLa cells using flow cytometry analysis, which measures the GFP fluorescence intensity of each cell. Transfection with fusion constructs containing *exoS*, *exoS(E381A)*, and *exoS(R146K/E381A)* resulted in 11.1, 74.7, and 73.3%, respectively, of total cells marked as GFP positive 24 h after transfection (Fig. 4B). In the GFP-signal-versus-cell-number histogram, these *egfp-exoS*-transfected cells showed substantially lower GFP values than those in the other two cases (Fig. 4B). Remarkably,

all EGFP-ExoSEA-expressing cells, but not EGFP-ExoS-RKEA-expressing cells, exhibited rounding morphology, suggesting an intact GAP activity in the fusion protein (Fig. 4C). Taken together, the majority of EGFP-ExoS-expressing cells died and lost the GFP signal rapidly, whereas most of the EGFP-ExoSE381A- and EGFP-ExoSR146KE381A-expressing cells stayed alive and maintained strong GFP signal over the time.

Furthermore, caspase-3 activation was examined in the transfected HeLa cells using Red-DEVD-FMK. EGFP-ExoS expression led to gradually enhanced caspase-3 activation, re-

FIG. 5. (A) Caspase-3 activation in EGFP-ExoS transfected HeLa cells. HeLa cells were transfected with EGFP fusion protein constructs as indicated. The resulting HeLa cells were collected 24 h after transfection, and a caspase-3 activation assay was performed using Red-DEVD-FMK as described in Materials and Methods. Flow cytometry analysis was performed using a FL-2 channel on a FACS sort cytometer. Dot density analysis of each sample is represented as the FL-2 intensity of a single cell (x axis) and the forward light scatter of the cells (y axis). The index for the gating population with activated caspase-3 in this assay was determined from the analysis pattern for HeLa cells treated with 2 μ M STS for 4 h, which served as a positive control (subpanel B). Remaining subpanels: A, CTRL, no DNA was added for the transfection procedure; C, EGFP-S, wild-type *exoS* in fusion construct; D, EGFP-EA, *exoS(E381A)* in fusion construct; E, EGFP, vector control; F, EGFP-RKEA, *exoS(R146K/E381A)* in fusion construct. (B) Caspase-3 activation in total transfected HeLa cells is reduced by pan-caspase inhibitor. Caspase-3 activation in HeLa cells was assayed 24 h after transfection with the indicated constructs or 3 h after STS treatment. HeLa cells were treated with Boc-D-fmk at 80 μ M immediately after transfection. The data are means $\pm$ the SD from three replicates from one transfection experiment. Significant differences between Boc-D-fmk-treated and untreated (CTRL) groups are shown (**, $P < 0.01$).

sulting in activated caspase-3 in 53.6% of the total cells or nearly 80% of the GFP-positive subpopulation by 24 h posttransfection, which was comparable to the 60.0% of HeLa cells at 3 h after STS treatment (2 μ M) (Fig. 5A and Table 2). STS treatment served as a positive control for the apoptosis induction. In contrast, at 24 h posttransfection, the expression of EGFP, EGFP-ExoSEA, and EGFP-ExoSRKEA resulted in caspase-3 activation in 13.8, 11.6, and 7.0% of total cells or 9.1, 9.2, and 4.1% among EGFP-positive populations, respectively (Fig. 5A and Table 2). Combining the data obtained at all

three time points posttransfection, in GFP-positive HeLa cells, the caspase-3 activity was consistently and significantly higher for transfection with EGFP-ExoS than with EGFP (Table 2). Importantly, treatment of HeLa cells with Boc-D-FMK, a cell-permeable broad-spectrum caspase inhibitor (22, 66), significantly reduced apoptosis elicited by the EGFP-ExoS, as judged by caspase-3 activity assay (Fig. 5B) and Hoechst staining assay (data not shown). In contrast, glycine, a necrosis inhibitor (51), failed to reverse the caspase-3 activation (data not shown), indicating an apoptosis-specific phenomenon that is not secondary to possible necrosis.

JNK signaling and the mitochondrion proapoptotic pathway were both required in EGFP-ExoS-induced apoptosis. Previously, we demonstrated that *P. aeruginosa* secreting ExoS

TABLE 2. Caspase-3 is activated in EGFP-ExoS-transfected HeLa cells^a

Time post-transfection (h)	Fraction	Mean content (%) $\pm$ SD ^b			
		EGFP	S	EA	RKEA
8	Total	3.8 $\pm$ 1.9	4.8 $\pm$ 1.6	3.7 $\pm$ 1.3	3.8 $\pm$ 1.5
	GFP ⁺	4.0 $\pm$ 0.9	26.6 $\pm$ 4.7*	4.1 $\pm$ 0.6	3.6 $\pm$ 0.8
12	Total	4.9 $\pm$ 2.8	10.9 $\pm$ 2.7	4.5 $\pm$ 1.4	3.6 $\pm$ 1.0
	GFP ⁺	5.7 $\pm$ 0.9	34.3 $\pm$ 3.6**	5.1 $\pm$ 0.7	2.9 $\pm$ 0.5
24	Total	13.8 $\pm$ 3.7	53.6 $\pm$ 10.8**	11.6 $\pm$ 2.1	7.0 $\pm$ 1.4
	GFP ⁺	9.1 $\pm$ 1.9	75.2 $\pm$ 6.9***	9.2 $\pm$ 0.6	4.1 $\pm$ 0.5

^a HeLa cells were transfected with EGFP fusion protein constructs as indicated. The resulting HeLa cells were collected at the indicated times after transfection, and a caspase-3 activation assay was performed using Red-DEVD-FMK as described in Materials and Methods. Flow cytometry analysis was performed using FL-1 and FL-2 channels on a FACSsort cytometer. Cells with activated caspase-3 were determined based on the FL-2 channel reading as described in Fig. 5. Cells with EGFP (GFP⁺) were determined based on the FL-1 channel reading as described in Fig. 4. Detection through both channels was adjusted based on signal-positive control (i.e., either without Red-DEVD-FMK staining, for FL-2 negative, or without EGFP transfection, for FL-1 negative, respectively) in order to minimize false positives. The percentages of cells with caspase-3 activation in transfected HeLa cells are shown as in total HeLa cells (Total) or in cells with detectable GFP signal (GFP⁺). EGFP, vector control; S, wild-type *exoS* in fusion construct; EA, *exoS(E381A)* in fusion construct; RKEA, *exoS(R146K/E381A)* in fusion construct. Data are means from three replicates. Significant differences between EGFP- and EGFP-ExoS-transfected groups are indicated as follows: *, $P < 0.05$; **, $P < 0.01$, and ***, $P < 0.001$.

triggers a proapoptotic pathway through JNK-mediated cytochrome *c* release from mitochondria (48). Here, we used the HeLa cells overexpressing DN JNK1 (48) to test the role of JNK in the EGFP-ExoS-induced apoptosis. Upon transient transfection with EGFP-ExoS, HeLa/DN JNK1 cells were much less sensitive to apoptosis induction than the HeLa/pcDNA3 (vector control) (Fig. 6A). Furthermore, overexpression of the antiapoptotic mitochondrial protein Bcl-x_L in HeLa cells (65, 69) significantly reduced caspase-3 activation after transfection with EGFP-ExoS (Fig. 6B). As a control in this experiment (Fig. 6B), STS-triggered apoptosis was also blocked by the overexpression of Bcl-x_L in a similar fashion (87). In agreement with this, HeLa/Bcl-x_L cells exhibited significantly reduced sensitivity to apoptosis induction by PAK infection compared to the HeLa/Neo control cell line, although there is no difference in terms of ExoS secretion efficiency for the involved cell lines (data not shown). These results demonstrate that both JNK activation and cytochrome *c* release from mitochondria play important roles in promoting apoptosis in the current model.

The cellular pathway(s) mediating ExoS-induced cytotoxicity is evolutionarily conserved. To test whether the molecular mechanism of ExoS-induced cell death is conserved, we expressed *exoS*, *exoS(E381A)*, *exoS(R146K)*, and *exoS(R146K/E381A)* in *Drosophila* S2 cells. Expression of ExoS using the *Pie3* insect virus promoter induced rapid cell death within 20 h after the transfection (Fig. 7A), displaying an efficiency similar to that induced by the expression of *reaper*, a proapoptotic gene in *Drosophila* (99). This result is not a total surprise. Previous studies have shown that *P. aeruginosa* infects *Drosophila* larvae and adults and that the TTSS is responsible for killing the organisms (3, 19, 24). We demonstrated here that the intracellular expression of ExoS alone is sufficient to induce

apoptosis in *Drosophila* cells. Similar to its effect on mammalian cells, ExoS-induced cytotoxicity in insect cells mainly depends on its ADPRT activity. The ADPRT-null mutation (E381A) abolished ExoS-induced cell death, whereas the GAP-null mutation (R146K) had little effect (Fig. 7A). The IAP and IAP-antagonist (Reaper) pathway play important roles in regulating cell death during insect development; IAP-antagonist, thus far, is the most potent proapoptotic regulator in insect cells (10). However, whereas the coexpression of DIAP1 (for *Drosophila* inhibitor of apoptosis protein 1) significantly suppressed IAP-antagonist-induced cell death, it had little effect on the ExoS-induced cell death (Fig. 7B), suggesting that either ExoS-induced cytotoxicity is not mediated through the IAP pathway or the direct target of ExoS is located lower in the signal transduction cascade than the level of IAP function in *Drosophila* cells.

Collectively, ExoS, either by itself or in the form of a chimeric protein, efficiently induces apoptosis in HeLa cells, as well as *Drosophila* S2 cells, independent of other factors from

FIG. 6. Caspase-3 activation in EGFP-ExoS transfected HeLa cells is dependent on JNK1 activation and cytochrome *c* release. (A and B) HeLa/pcDNA3 (vector) or HeLa/DN JNK1 cells (A) and HeLa/Neo vector (vector) or HeLa/Bcl-x_L cells (B) were transfected with the EGFP-ExoS construct and subjected to caspase-3 activation assay with Red-DEVD-FMK as described above. As a control for apoptosis induction, STS (2 μ M) was used to treat the indicated cells for 3 h. The data are means $\pm$ the SD from three replicates from one transfection experiment. Significant differences between HeLa/pcDNA3 (vector) or HeLa/DN JNK1 cells (A) or between HeLa/Neo vector (vector) and HeLa/Bcl-x_L cells (B) are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

FIG. 7. (A) ExoS induced cell death in *Drosophila* S2 cells. Similar to its effect on mammalian cells, the cytotoxicity of ExoS in *Drosophila* S2 cells is dependent on its ADP-ribosylating activity (EA, ExoS E381A; RK, ExoS R146K; RKEA, ExoS R146K/E381A). Wild type and ExoS RK killed essentially 100% transfected cells, whereas the ExoS EA and ExoS RKEA double mutants have dramatically reduced cytotoxic activity. (B) While increasing the level of dIAP1 significantly suppressed reaper-induced cell death, it had little effect on ExoS-induced cytotoxicity. The colors of the bar reflect the relative ratio between proapoptotic gene constructs and Diap1. For both panels A and B, the data are represented as averages ($n = 5$ and 4 , respectively), and error bars indicate the SD.

P. aeruginosa. This induction correlates directly with its functional ADPRT domain but not the GAP domain. Also importantly, the apoptotic signal transduction events that are involved in the current ExoS transfection model, such as the essential roles played by JNKs, mitochondria, and caspases, mirror the previously described pathway wherein apoptosis was triggered upon infection by ExoS-secreting *P. aeruginosa* (48).

DISCUSSION

ExoS, along with other *P. aeruginosa* type III secretion effectors, has been shown to be an important virulence determinant in various animal infection models (42, 57–59, 83). In addition, most cystic fibrosis (CF) patients harbor antibodies to ExoS (4). On the other hand, clinical isolates from CF patients have also been shown to predominantly lack the ability to secrete type III effector molecules, including ExoS (20, 80). Indeed, ExoS plays an important role in the initial colonization and persistence in the CF lung but not during the chronic stage of the disease (59, 83). It was further suggested that the CF strains accumulate a number of mutations to ensure the reduced production of type III effectors and therefore bacterial toxicity to the host (59). Nonetheless, it remains a partially elucidated picture how the “on and off” switch of ExoS secretion in susceptible human host correlates with the progression

of the *P. aeruginosa* infection. In recent years, increasing numbers of studies, both in vitro or in vivo, describe apoptosis induction by *P. aeruginosa* (17, 23, 36, 38, 46, 77, 89); some of the studies identified a variety of apoptosis-inducing factors from the bacterium (2, 7, 8, 34, 47, 50, 88). Bruno et al. (7) and we (50) first reported ExoS as one of these factors. The present study stems from our previous observations, directly correlating the apoptosis induction with the ADPRT-competent ExoS independent of any other bacterial factor.

Our attempts to generate stable HeLa cell lines with inducible expression of wild-type ExoS were not successful. The failure is likely due to a leakiness of the control of transcription in the selected system, a finding similar to those observed by others (94). Indeed, trace amounts of ExoS were detected in transfectants even in the absence of inducer. However, an elevated expression of *exoS* was achieved in the transfectants that were transiently transfected using the same system, and the resulting expression of ExoS, which was confirmed by Western blotting (data not shown), led to a substantial increase in apoptotic cells, as assessed by both caspase-3 activation and nucleus condensation, whereas that of ExoS E381A failed to do so.

A GFP reporter, carried either by pUF5 or pEGFP-C1, was used as an indicator of successful transfection in analysis of ExoS-mediated apoptosis in present study. The total GFP signal intensity in the cell is affected by the rate of de novo protein synthesis and the stability of the protein, both of which are likely affected during the ExoS-induced apoptosis process. This is evident in the cotransfection experiment, in which, although strong blockage of caspase-3 activation and nuclei condensation were achieved upon application of a caspase inhibitor (Fig. 1), the GFP signal was only partially restored in the transfected population (Fig. 1 and data not shown), suggesting that exogenously expressed ExoS affects protein synthesis and/or protein stability in the cell in addition to triggering apoptosis. Apoptosis-related inhibition of translation has been shown in an array of cell lines, including HeLa cells (12, 63). In particular, caspase-3-independent degradation of PABP [for poly(A)-binding protein] has been reported (63). Nevertheless, the effect on translation should not be the direct cause of the efficient apoptosis induction demonstrated in our study, since the inhibition of translation in HeLa cells by CHX alone did not lead to rapid apoptosis (data not shown; see also reference 6); it is rather the result of rapid self-deterioration of apoptotic cells. Moreover, this effect should not be significantly contributed by the GAP activity of the ExoS since transfection featuring ExoS E381A led to most, if not all, of the resulting cells being EGFP positive (Fig. 4B), indicating maintenance of the cellular protein level, despite the dramatic alteration of the cellular morphology resulting from the intact GAP activity (25, 32, 54).

Interestingly, in the experiments with EGFP chimeric proteins, an extensive degree of degradation was observed for all derivatives of full-length chimeric proteins (Fig. 3), suggesting that the degradation of EGFP fusion protein is at least partially triggered by an ExoS-specific proteasome pathway. It is worth noting that, when treated with Boc-D-FMK, a general caspase inhibitor, EGFP-ExoS-transfected HeLa cells were only marginally (although noticeably) rescued for the GFP signal (data not shown), whereas cells that were cotransfected

with GFP and ExoS (i.e., the two proteins were expressed independently) displayed much more significant restoration of the signal (Fig. 1), suggesting a possible ExoS-associated degradation. Type III toxins secreted by *Salmonella enterica* reportedly undergo proteasome-dependent protein degradation, which plays an essential role in the temporal regulation of their function (56). It will be interesting to evaluate and confirm the possible degradation of ExoS in future studies.

After injection into the host cell cytosol through the TTSS of *P. aeruginosa*, ExoS has been shown to localize to the perinuclear region within host cells due to the function of a membrane localization domain localized between amino acid residues 51 and 72 (55, 71, 72). The EGFP-transfected HeLa cells, as previously well-documented (79), display both cytoplasmic and nuclear fluorescence, whereas in their EGFP-ExoS381A and EGFP-ExoS146KE381A counterparts, the fluorescence appeared to be excluded from nuclei (Fig. 4C), which closely resembles the pattern of perinuclear localization of the ExoS mediated by the membrane localization domain (55, 97). Therefore, the subcellular distribution pattern and GAP domain of the chimeric proteins remained intact, and the cell death data presented above also suggested that the ADPRT domain in EGFP-ExoS retained its catalytic function and targeting specificity as well.

Furthermore, it has been documented that cells in different phases of the cell cycle have different sensitivities to apoptosis induction (21). Therefore, it is conceivable that in the EGFP-ExoS transfection experiments, the variation of EGFP intensity in each cell may reflect a cell cycle-dependent sensitivity to ExoS-induced apoptosis. In those less-sensitive cells, the proapoptotic signal needs to overcome a higher threshold in order to push the balance toward apoptosis. As a result, more EGFP-ExoS fusion proteins are accumulated before apoptosis is triggered in these cells, and thus the cells displayed a higher intensity of GFP. In the EGFP-ExoS-transfected HeLa cells, nearly 54% of the total population was apoptotic (Fig. 5A), which correlates with a consistent >60% frequency of transfection. However, only 11% of the total cells displayed detectable EGFP; thus, we believe that the majority of the transfected, yet EGFP signal-negative HeLa cells underwent apoptosis. Considering ca. 60% of the STS-treated HeLa cells were apoptotic, 80% of the GFP-positive subpopulation undergoing apoptosis (Fig. 5C) is highly significant for EGFP-ExoS-transfected cells. These data strongly suggested that the appearance of GFP signal in these cells is an indication of their temporal insensitivity to apoptosis induction, possibly due to a higher tolerance at a certain cell cycle state. Studies are under way to test this possibility.

The apoptosis induction, as a result of the expression of either ExoS alone or EGFP-ExoS, appeared to be attenuated compared to that resulting from *P. aeruginosa* infection (48, 50). The rate of apoptosis elicitation was slower, and the intensity was reduced in terms of caspase-3 activation. In contrast to the direct injection of ExoS into HeLa cells by the bacteria at a fast and uniform rate, the uptake of exogenous DNA and its expression are much more time and energy consuming, which might be responsible for the reduced tempo and intensity of the apoptosis induction.

It remains obscure how the ADPRT activity of ExoS leads to apoptosis in host cells. The ADPRT of ExoS shares a similar

motif with the arginine-specific mono-ADP-ribosyltransferase of eukaryotic cells (96). Endogenous mono-ADPRT of proteins has not been associated with the apoptotic process. However, pretreatment with a potent inhibitor of arginine-specific mono-ADPRT has been demonstrated to suppress TNF-induced DNA fragmentation in U937 cells in a dose-dependent manner (90). In addition, cytosolic mono-ADPRT activity is elevated in cells undergoing apoptosis (91). These data support the hypothesis that mono-ADPRT may function in apoptotic signal transduction. On the other hand, it has also been speculated that the hyperactivity of ADPRT may deplete the cellular levels of NAD, and subsequently ATP, which ultimately causes cell death due to impaired energy metabolism (9).

A great deal of progress has been made in defining the biological targets of ExoS in host cells. ExoS recognizes a wide variety of genetically or structurally diverse proteins for ADPRT, including the monomeric GTPases, vimentin, and other undefined host proteins (6, 13, 62). Most dominant substrates are Ras proteins, especially H-Ras, N-Ras, and K-Ras, three proteins that are ubiquitously expressed in mammalian cells (26, 41). Ras and several related proteins, including Rab3, Rab4, Rab5, Ral, Rap1A, and Rap2, were identified as targets of ExoS (15, 16, 26, 41). The ADPRT of these components by ExoS disrupted signaling by inhibiting guanine nucleotide exchange factor-catalyzed nucleotide exchange, which uncoupled respective signal transduction (5, 29, 30, 78). Ras and Ral, both important in the proliferation and survival of the host cell and ADP-ribosylated by ExoS, represent the antiapoptosis barrier that ExoS ADPRT will have to overcome to induce apoptosis. Most recently, Jansson et al. (45) reported that when *Yersinia pseudotuberculosis* TTSS was used as a tool to deliver ExoS proteins into fibroblast and kidney cells, activated Ras was shown to protect infected cells against ExoS-induced apoptosis, regardless of whether activated Ras was modified by ExoS. We have previously drawn a direct link between ExoS ADPRT domain and its ability to trigger the proapoptotic signaling through a JNK/mitochondrion/caspase-3 pathway that is facilitated by the inhibition of the Erk survival pathway (48, 50). Differential sensitivities of the two signaling pathways to ExoS might explain the cell type-dependent sensitivity to the killing by *P. aeruginosa* (14). Two critical questions remain unanswered. (i) Is the ExoS-mediated blockage of Ras signaling sufficient to trigger host cell apoptosis? (ii) If it is sufficient, how does the disruption of Ras signaling warrant JNK activation and the downstream proapoptosis pathway? If not, what else is involved? We should be able to better define the unique involvement of ExoS ADPRT in apoptosis induction by comparing ExoS with other known endogenous mono-ADPRTs with regard to intracellular processing, cofactor binding, and in vivo substrate targets.

Even though the nature of the cellular pathway(s) mediating ExoS induces cell death remains elusive, it is clear that the responsible pathway(s) is genetically conserved. We have shown that ExoS, when expressed, induces rapid cell death in *Drosophila* S2 cells. Similar to its effect on mammalian systems, the cytotoxic activity of ExoS is mainly dependent on the ADPRT activity. Intriguingly, identification of the putative target(s) of ExoS in S2 cells may very likely provide a great deal of information that helps elucidate its role in apoptosis induction in mammalian systems. These findings paved the way for

using the powerful genetic system offered by the fruit fly to conduct systematic searches for the cellular targets mediating ExoS-induced cytotoxicity.

Overall, by using two approaches to express ExoS in HeLa cells, we demonstrate that ExoS alone is sufficient to mediate apoptosis in host cells. Furthermore, the ADPRT of ExoS is the key factor for initiating the proapoptotic signaling pathways in host cells. Therefore, TTSS secreted ExoS serves as an effective apoptosis-inducing agent for *P. aeruginosa* to manipulate the fate of host cell and to promote its prominent infection cycle in susceptible host. Finally, with ongoing study to fully elucidate the cellular mechanism for its action, ExoS presents a great deal of potential as an apoptosis-inducing molecule that may be tested as a novel cancer therapeutic agent in the future.

ACKNOWLEDGMENTS

We thank Alfred Lewin for the gift of pUF5, Xiaodong Wang for the HeLa-Bcl-x_L cell lines, Lung-Ji Chang for critical support, Neal Benson and Bhavna Bhardwaj (ICBR, University of Florida) for assistance on flow cytometry, and Carl P. Santos for technical assistance.

This study was supported by a Research Scholar Award from the American Cancer Society (to S.J.) and a National Institutes of Health grant (to L.Z.).

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Editor: J. B. Bliska