c-Jun NH₂-Terminal Kinase-Mediated Signaling Is Essential for *Pseudomonas aeruginosa* ExoS-Induced Apoptosis

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As an opportunistic bacterial pathogen, *Pseudomonas aeruginosa* mainly affects immunocompromised individuals as well as patients with cystic fibrosis. In a previous study, we showed that ExoS of *P. aeruginosa*, when injected into host cells through a type III secretion apparatus, functions as an effector molecule to trigger apoptosis in various tissue culture cells. Here, we show that injection of the ExoS into HeLa cells activates c-Jun NH₂-terminal kinase (JNK) phosphorylation while shutting down ERK1/2 and p38 phosphorylation. Inhibiting JNK activation by expression of a dominant negative JNK1 or with a specific JNK inhibitor abolishes ExoS-triggered apoptosis, demonstrating the requirement for JNK-mediated signaling. Following JNK phosphorylation, cytochrome *c* is released into the cytosol, leading to the activation of caspase 9 and eventually caspase 3. Although c-Jun phosphorylation is also observed as a result of JNK activation, ongoing host protein synthesis is not essential for the apoptotic induction, suggesting that c-Jun- or other AP-1-driven activation of gene expression is dispensable in this process. Therefore, ExoS has opposing effects on different cellular pathways that regulate apoptosis: it 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. These results highlight the modulation of host cell signaling by the type III secretion system during interaction between *P. aeruginosa* and host cells.

The opportunistic bacterial pathogen *Pseudomonas aeruginosa* causes devastating human infections in patients with cystic fibrosis, severe burns, or immunosuppression (58). To successfully colonize and maintain its infectious cycle, *P. aeruginosa* carefully orchestrates production of a suite of virulence determinants. Some of the virulence factors are produced and secreted directly into the host cell using the cell contact-mediated type III secretion machinery (9, 36). To date, four such factors have been characterized in *P. aeruginosa*, including the ADP-ribosylating enzymes, ExoS and ExoT (18, 20, 65); an acute cytolytic factor, ExoU (17, 28); and an adenylate cyclase, ExoY (66). Expression of these secreted effector molecules along with components of the type III secretory apparatus is under the control of a transcriptional activator, ExsA, which responds to low calcium and cell contact signals (35).

The two ADP-ribosylating toxins, ExoS and ExoT, share 75% amino acid identity, yet ExoT possesses only 0.2% of the ADP ribosyltransferase (ADPRT) activity of ExoS (18). The ADPRT activity of ExoS has been shown to modify Ras and several Ras-like host proteins in vivo, including Rab5, RalA, and Rap (2, 3, 19, 52). ADP ribosylation of Ras by ExoS has been linked to the disruption of Ras-Raf-mediated signaling in vitro and in vivo (22, 30, 61). The catalytic activity of ExoS is dependent on eukaryotic host protein(s) termed FAS (for factor activating ExoS), which is a member of the 14-3-3 protein

family (21, 70). In addition to the ADPRT activity located in the C-terminal domains of ExoS and ExoT, the N-terminal domains of these two toxins display GTPase-activating protein (GAP) activity for the small-molecular-weight GTPases Rho, Rac, and Cdc42 (23). Once translocated into host cells, ExoS localizes to the perinuclear region and then is processed into a soluble fragment that retains both GAP and ADPRT activities (49).

Among the family of small GTPases, the subfamily of Ras and Rho proteins hierarchically and/or coordinately regulate signaling pathways that control critical processes such as proliferation, differentiation, and apoptosis (55). In addition, depending on the cell type and experimental system, Ras proteins have been implicated in both the promotion and prevention of apoptosis (16, 51). These apparently contradictory functions can be explained by the ability of Ras to regulate multiple signaling pathways via interaction with different effector molecules, such as the mitogen-activated protein kinases (MAPK) (51). Three MAPK subgroups have been identified in mammalian cells: ERK (extracellular signal-regulated kinase), JNK (c-Jun NH₂-terminal kinase), and p38 (63). MAPKs are activated by sequential phosphorylation in the context of a three-kinase (MAPKKK/MAPKK/MAPK) module. A major role of ERK is to respond to growth factor stimulation, where ERK-regulated signaling is activated through Ras-dependent pathways; Ras effectors, including Raf-1, the aPKCs (atypical PKCs), and PI 3-kinase, activate MEK1/2, which in turn activates ERK (6, 13, 14, 53). JNK and p38 are activated most strongly by environmental stress stimuli (UV radiation, heat shock, osmotic shock, protein synthesis inhibitors) and by

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties ^a	Reference or source
<i>P. aeruginosa</i>		
PAK	Clinical isolate of wild-type invasive strain	D. Bradley
PAK <i>exoS</i>	PAK with chromosomal disruption of the <i>exoS</i> locus; Sp ^r Sm ^r	41
PAK <i>exoS</i> <i>exoT</i>	PAK with chromosomal disruption of the <i>exoS</i> and <i>exoT</i> loci; Sp ^r Sm ^r Gm ^r	41
Plasmids		
pCR2.1-TOPO	PCR cloning vector; Ap ^r Km ^r	Invitrogen
pUCP18	Broad host range shuttle vector; Ap ^r	54
pUCP <i>exoS</i>	<i>exoS</i> gene derived from PAK in pUCP18; Ap ^r	Unhwan Ha
pUCP <i>exoT</i>	<i>exoT</i> gene derived from PAK in pUCP18; Ap ^r	Unhwan Ha
pUCP <i>exoSE381A</i>	<i>exoS</i> of pUCP <i>exoS</i> with single AA mutation at E381A; Ap ^r	41

^a Antibiotic resistance markers: Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Sp^r, spectinomycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance.

proinflammatory cytokines (e.g., interleukin-1 and tumor necrosis factor alpha [TNF- α]). Several MAPKKs, including MEKK1 (67), ASK1 (38) and MLKs (33), have been identified which, when activated, subsequently activate one of two MAPKKs, namely, MKK4 (67) or MKK7 (44), which can directly activate JNK. The p38, on the other hand, is phosphorylated and activated by either MKK3 (12) or MKK6 (26). ERK and JNK often have opposing functions in apoptosis, with a balance-of-growth factor-activated ERK and stress-activated JNK dictating the outcome after an apoptotic stimulus (64). To complicate matters more, JNK signaling and downstream c-Jun/AP-1 have been implicated in various and often opposing cellular responses, including proliferation, differentiation, and apoptosis; the effects of JNK on cellular responses appear to depend on the cell type and the context of other signals received by the cell (32). Recent studies with JNK isoform knockout cells have clearly demonstrated that JNK is required for neuronal apoptosis in response to excitotoxic stress and for UV-induced apoptosis of murine embryonic fibroblasts (56, 69). However, the mechanisms involved in JNK-mediated apoptosis have remained mostly obscure.

Previously, it has been documented that type III secreted exotoxin ExoS triggers apoptosis in various host cells upon infection by invasive *P. aeruginosa* strains (7, 41). It was further demonstrated that the ADPRT activity of the ExoS is responsible for triggering the apoptosis (41). In addition, *P. aeruginosa* has also been shown to induce apoptosis in macrophages, T lymphocytes, mouse airway epithelial cells, and human endothelial cells (24, 27, 50, 59). In this study, we examine the role of MAPKs in the signal transduction pathway leading to host cell apoptosis in response to ExoS of *P. aeruginosa*. We demonstrate that ExoS not only triggers a proapoptotic pathway through JNK-mediated cytochrome *c* release but also sensitizes the host cell to proapoptotic signals by inhibiting antiapoptotic pathway(s) controlled by ERK1/2 and possibly p38. Based on these data, a hypothetical pathway leading the host cell to apoptosis in response to the ExoS has been proposed.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown in Luria-Bertani broth at 37°C. For site-specific mutagenesis, the method of Kunkel et al. (43) was used.

Materials. Anti-JNK1 (1:500) and anti-actin (1:500) antibodies, used at the indicated dilutions, were obtained from Santa Cruz Biotech (Santa Cruz, Calif.).

Anti-p-JNK (1:2,000), anti-p38 (1:1,000), anti-p-p38 (1:1,000), anti-p-ERK1/2 (1:1,000), anti-ERK1/2 (1:1,000), anti-p-c-Jun (at Ser63; 1:2,000) and anti-caspase 9 (1:1,000) antibodies were purchased from Cell Signaling Inc. (Beverly, Mass.). Anti-cytochrome *c* antibody was purchased from Pharmingen (BD Biosciences, San Diego, Calif.). Peroxidase-conjugated goat anti-rabbit secondary antibodies and peroxidase-conjugated goat anti-mouse antibodies were from Santa Cruz Biotech and Amersham Pharmacia Biotech (Piscataway, N.J.), respectively. PD98059, U0126, SB203580, SP600125, staurosporine, cycloheximide (CHX), and caspase 9 inhibitor Z-LEHD-FMK were obtained from CalBiochem (San Diego, Calif.).

Generation of HeLa cell lines stably expressing dominant-negative JNK. Cells were plated on six-well plates at 1.5×10^5 cells/well in Dulbecco's modified Eagle medium (DMEM)–10% fetal calf serum (FCS), 100 units 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 dominant-negative JNK construct pcDNA3/DN-JNK1 (11) or pcDNA3 vector with LipoFectamine-Plus reagent (Life Technologies Inc.). 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/ml (Life Technologies Inc.).

Infection of HeLa cells by *P. aeruginosa*. HeLa S3 cells in suspension culture were maintained in Joklik's modified minimal essential medium supplemented with 100 units of penicillin G/ml, 100 μ g of streptomycin/ml, and 7.5% horse serum (MEM + 7% HS) (Gibco). HeLa cell monolayers were plated from suspension culture 1 day prior to infection in DMEM–5% FCS (Gibco). HeLa cell monolayers ($\sim 5 \times 10^5$ cells per well; >80% confluence) were washed with phosphate-buffered saline (PBS), inoculated with bacteria (10^7 CFU/ml of DMEM; multiplicity of infection, 20), and incubated for 2 h at 37°C in a 5% CO₂ incubator. The cells were washed with PBS to remove the nonadherent bacteria. Fresh medium, consisting of DMEM–5% FCS supplemented with 400 μ g of gentamicin/ml or 400 μ g of amikacin/ml, was added, and the cell monolayers were incubated for an additional 3 to 24 h. As positive controls for the apoptosis, HeLa cells were incubated with 10 ng of TNF- α /ml and 20 μ g of CHX/ml (TNF- α –CHX), or with 2 μ M staurosporine (STS). In some experiments, as indicated, SP600125 (25 or 50 μ M) or CHX (25 μ M) was added into fresh medium of HeLa cells at 30 min or 2 h, respectively, before bacterial infection. The specific kinase inhibitors, U0126 (20 μ M), PD98059 (60 μ M), or SB203580 (10 μ M), were added into culture for 5 h before cells were processed for apoptosis assay. In caspase 9 inhibition experiments, the caspase 9 inhibitor Z-LEHD-FMK was added into culture 2 h before starting infection. There was no noticeable difference in terms of binding of bacteria to host cells among all the strains used in this study (reference 41 and data not shown).

Caspase 3 activity assay. Caspase 3 activity was measured with a Caspase 3 Cellular Activity Assay Kit Plus (BioMol). HeLa cells (3×10^7) were infected with *P. aeruginosa*, and then cells were washed and harvested by scraping and centrifugation ($1,000 \times g$ for 10 min) at various times postinfection (p.i.). Cells were lysed with 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 optical density at 405 nm were followed for 2 h at 10-min intervals. Protein concentrations were determined with a protein assay system from Bio-Rad. The specific activity is reported as picomoles of substrate cleaved/minute per microgram of protein.

FIG. 1. ExoS of PAK alters MAPK activation in HeLa cells. HeLa cells were infected with PAK, PAK*exoST*/pUCP18*exoS* (ExoST/S), or PAK*exoST*/pUCP18*exoSE381A* (ExoST/EA) or left uninfected as control (lanes C). At the indicated times p.i., cell lysates were prepared and analyzed by Western blotting. The same samples were blotted with antibody against Phospho-JNK or JNK1/2 (A), Phospho-p38 or p38 (B), and Phospho-ERK1/2 or ERK1/2 (C). All membranes were reprobed against β -actin.

Western blot analysis of JNK, p38, and c-Jun. At different times p.i., HeLa cell pellets were suspended in lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS) (wt/vol), 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue. Equal amounts of total protein were subjected to 12% SDS-polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, proteins were transferred to PVDF-Plus membranes (Osmonics Inc., Minnetonka, Minn.) at 50 mA for 60 min by using a semidry protein transfer system (Bio-Rad). Blots were blocked with 5% (wt/vol) dry milk in 1 $\times$ Tris-buffered saline (TBS) buffer (50 mM Tris-HCl, 150 mM NaCl [pH 7.4])–0.5% (vol/vol) Tween 20 for 60 min and probed with the appropriate antibodies for 1 h at room temperature or overnight at 4°C, in accordance with suppliers' recommendations. After being washed with 1 $\times$ TBS–0.1% Tween 20, membranes were incubated with peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies for 60 min. Specific signals were developed using the ECL-Plus system (Amersham). To ensure equal loading and even transfer of proteins, protein bands were visualized by staining the membranes with Ponceau S (0.1% Ponceau S in 3% trichloroacetic acid, wt/vol) and further reprobed with anti- β -actin antibody after treatment for 20 min at 60°C with stripping buffer containing 62.5 mM Tris-HCl (pH 6.7), 100 mM β -mercaptoethanol, and 2% (vol/vol) SDS. The bands were quantified with the densitometry program LabWorks (UVP Inc., Upland, Calif.). All measurements of phosphorylated kinase and total kinase protein levels were normalized against the anti- β -actin signal. Each Western blotting experiment was conducted with two separate membranes in parallel to ensure reproducibility.

Detection of cytochrome *c* release. Cytosolic proteins of the HeLa cells were obtained by a digitonin-based subcellular fractionation technique, essentially as described previously (1). Briefly, at various times p.i., infected HeLa cell monolayers were scraped and the cells were pelleted by centrifugation at 700 $\times$ g for 5 min. The cell pellet was washed twice with ice-cold PBS, resuspended in cytosolic extraction buffer (3 $\times$ 10⁶ cells/0.2 ml; 200 μ g of digitonin/ml, 250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄ [pH 7.2], 100 μ M phenylmethylsulfonyl fluoride, 10 μ g of leupeptin/ml, 2 μ g of aprotinin/ml), and incubated on ice for 5 min. Plasma membrane permeabilization of cells was confirmed by staining in 0.2% trypan blue solution. The samples were then centrifuged at 1,000 $\times$ g for 5 min at 4°C, and the supernatant was collected as the cytosolic fraction. For the detection of cytochrome *c*, equal

volumes (20 μ l) of cytosolic fractions were separated by 15% SDS-polyacrylamide gel electrophoresis and analyzed by Western blots with anti cytochrome *c* antibodies.

RESULTS

ExoS activates JNK phosphorylation and blocks p38 and ERK1/2 phosphorylation in HeLa cells. As described previously (41), invasive strains of *P. aeruginosa* cause HeLa cell apoptosis in an ExoS-dependent manner. By using human cDNA microarrays to monitor gene expression profiles of HeLa cells following infection by either wild-type PAK or an *exoS* mutant (PAK*exoS*), we found that the MAP kinase pathways were affected by the ExoS (data not shown).

To examine the activation status of the three subgroups of MAPKs following infection by *P. aeruginosa*, we infected cells with either wild-type PAK, PAK/pUCP18, PAK*exoST*/*exoS*, or PAK*exoST*/*exoSE381A*. In the control experiments, wild-type PAK and PAK/pUCP18 exhibited the same host cell binding ability, apoptosis-inducing ability, and status and extent of phosphorylation of MAPKs (data not shown). The *exoSE381A* point mutation results in ADPRT-defective *exoS* that still retains its GAP activity (60). In addition, we have shown that apoptotic induction was dependent on the ADPRT but not the GAP activity of ExoS (41). Thus, any differences in MAPK phosphorylation detected in PAK*exoST*/*exoS*- and PAK*exoST*/*exoSE381A*-infected cells likely result from the ADPRT activity of ExoS and may be related to the apoptosis null phenotype of PAK*exoST*/*exoSE381A*. We analyzed the lysates from these

FIG. 2. HeLa cells expressing dominant negative JNK1 are resistant to the ExoS-mediated apoptosis. (A) HeLa cells were stably transfected with Flag-tagged dn JNK1 (pDNJNK1) or pcDNA3 vector plasmids (Vec). Cell lysates from three representative clones of pDNJNK1 (no. 1, 2, and 3, as indicated) and one pcDNA3 clone were subjected to immunoblot analysis with antibody against JNK1. (B) pDNJNK1 clone no. 1 and pcDNA3 clone were infected with PAK, and caspase 3 activities were measured at different times p.i. Samples were assayed in triplicate. *P* values (Student's *t* test) for comparing the infected and uninfected samples at each time point were as follows: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

infected cells for the phosphorylated or nonphosphorylated forms of JNK, p38, or ERK (Fig. 1).

JNK1 and JNK2 protein levels in uninfected or infected cells remained fairly constant over the first 4 h p.i. (Fig. 1A). This is consistent with the gene array data, which showed that both JNK1 and JNK2 mRNA levels remained unchanged up to 4 h p.i. (data not shown). Uninfected HeLa cells expressed both JNK1 and JNK2 isoforms in an unphosphorylated state, as assessed by immunoblot analysis with phospho-JNK antibodies (Fig. 1A). However, infection with either PAK or PAK*exoST/exoS* induced JNK1/2 phosphorylation that is evident by 2 to 4 h p.i. In contrast, infection with PAK*exoST/exoSE381A* did not lead to JNK phosphorylation (Fig. 1A). JNK activation appears to occur prior to caspase 3 activation (41), suggesting a possible regulatory role for JNK in apoptosis.

In contrast to JNK, p38 phosphorylation was significantly suppressed after infection by either PAK or PAK*exoST/exoS*, while infection by PAK*exoST/exoSE381A* resulted in increased p38 phosphorylation (Fig. 1B). These data indicate that p38-mediated signaling does not contribute to the ExoS-mediated apoptosis. Similarly, basal phosphorylation of ERK1/2, seen in uninfected control cells, was completely blocked at 2 h following infection by PAK (Fig. 1C). However, interruption of p38 or ERK1/2 activation with selective inhibitor U0126 or SB203580, respectively, did not induce apoptosis in uninfected HeLa cells within the same time frame (data not shown), indicating that the suppression of p38- or ERK1/2-mediated signal transduction seen in infected cells is not sufficient in and of itself to induce PAK-mediated apoptosis. It was also notable that protein levels of ERK1/2 decreased upon infection with the PAK derivatives (Fig. 1C); thus, down regulation of ERK

signaling may result from reductions at both the protein level and phosphorylation level. Interestingly, although to a lesser extent, these modifications of ERK signaling occurred with PAK*exoST/exoSE381A* infection as well (Fig. 1C), further suggesting that suppression of proliferating signals by the ERK1/2 pathway is not dependent solely on the ADPRT activity of ExoS. In addition, the absence of apoptotic induction in PAK*exoST/exoSE381A*-infected cells supports the interpretation of the pharmacologic data with p38 and ERK inhibitors in uninfected cells that suppression of both ERK1/2 and p38 pathways is not sufficient to induce apoptosis in HeLa cells. However, it remains plausible that PAK-mediated interruption of ERK1/2 signaling can modulate the proapoptotic JNK signaling pathway.

JNK-mediated signaling is essential for the ExoS-induced apoptosis. To evaluate the requirement of the JNK-mediated signaling in the apoptotic process, we generated a HeLa cell line stably transfected with a plasmid expressing the dominant-negative (dn) *jnk1* gene (10, 11). Immunoblot analysis of the cell lysates from the resulting HeLa cell clones confirmed that exogenous dnJNK1 protein was present in much higher abundance than the endogenous JNK1 protein (Fig. 2A). The apparent higher molecular weight of the dnJNK1 protein results from a C-terminal Flag-tag fusion. As shown by caspase 3 activation (Fig. 2B), the HeLa/dn *jnk1* cells were more resistant to PAK-mediated apoptosis, whereas pcDNA3 vector-transfected HeLa cells remained as sensitive as untransfected HeLa cells. The PAK bound equally well to the two HeLa-derived cell lines (data not shown). This demonstrated that JNK-mediated signaling is essential for the PAK-induced apoptosis.

FIG. 3. Activation of JNK1 is essential for the ExoS-mediated apoptosis. (A) HeLa cells were pretreated with 50 μ M SP600125 and then infected with PAK. At 4 h p.i., cell lysates were prepared for immunoblot analysis. The samples were blotted with antibody against phospho-JNK and JNK1. All membranes were reblotted with β -actin. Control samples are uninfected cells; SP, SP600125. (B) Cells were pretreated with various concentrations of SP600125 and infected with PAK. At 5 h p.i., caspase 3 activity was measured in infected and uninfected cell lysates. To indicate the significant difference from the control samples, Student's *t* test was performed for pair comparisons between the untreated (0 μ M SP) and SP-treated groups to determine *P* values: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

The above results were further confirmed by using a novel JNK-specific inhibitor, SP600125 (5). In uninfected HeLa cells, SP600125 inhibits JNK activation by anisomycin, a strong JNK activator (data not shown). Upon infection with PAK, phosphorylation of JNK1/2 was greatly reduced in SP600125-pretreated HeLa cells in comparison to nonpretreated HeLa cells (Fig. 3A). This reduction in JNK activation resulted in significantly reduced levels of caspase 3 activation in PAK-infected HeLa cells (Fig. 3B), strongly supporting a role of JNK in the PAK-mediated apoptosis. The reduction of caspase 3 activity displayed a dose dependence on SP600125 concentrations (up to 50 μ M); at concentrations greater than 50 μ M, severe toxicities were observed in the HeLa cells (data not shown).

Activation of c-Jun-regulated transcription is not required for ExoS-induced apoptosis. JNK may contribute to apoptosis induction either by transcription-dependent apoptotic signaling via c-Jun/AP-1 or by transcription-independent apoptotic signaling via phosphorylation-dependent posttranslational proapoptotic processes (cytochrome *c* release, stabilization of p53 protein, inactivation of Bcl-2 or Bcl-XL, or activation of c-myc) (10, 45, 46, 68). We next examined whether JNK-activated transcription-dependent or -independent signaling processes were required for induction of apoptosis.

Once activated, JNK is known to phosphorylate c-Jun, which

regulates the transcription of specific sets of genes, leading to a variety of responses (10). After infection of HeLa cells with the PAK derivatives described above, the status of c-Jun was examined by immunoblot analysis. As shown in Fig. 4A, significant increases in c-Jun phosphorylation were observed following infection with PAK or PAKexoST/exoS but not with PAKexoST/exoSE381A. Levels of total c-Jun protein in PAKexoST/exoS-infected HeLa cells increased more than levels in those infected with PAKexoST/exoSE381A over the same period of the time (Fig. 4A), which is consistent with the known ability of c-Jun to regulate its own expression (10).

Since c-Jun exerts its effect through activation of gene expression, inhibition of protein synthesis effectively blocks c-Jun-mediated transcription-dependent apoptotic signaling. Inhibition of host protein synthesis was achieved by pretreatment of HeLa cells with CHX, as described by Tournier et al. (56). This treatment produced no significant cytotoxicity in HeLa cells within the time frame of our assay. No difference in the timing or intensity of the apoptosis was observed in CHX-treated cells following PAK infection as judged by caspase 3 activity (Fig. 4B). Thus, inhibition of de novo protein synthesis does not interfere with PAK-mediated apoptosis, indicating that JNK promotes the apoptosis through transcription-independent apoptotic signaling, a mechanism that does not require transcriptional activation through c-Jun.

JNK activation correlates with cytochrome *c* release and caspase 9 activation. JNK can mediate proapoptotic signaling via modification of mitochondrial membrane proteins to enhance mitochondrial release of cytochrome *c* into the cytoplasm and subsequent activation of caspase 9 (56, 62). Release of cytochrome *c* into the cytosol results in activation of the caspase adaptor Apaf-1 and procaspase 9, forming a holoenzyme complex termed the apoptosome. Caspase 9 in the context of this holoenzyme activates downstream caspases, most importantly caspase 3 but also caspase 8, resulting in DNA fragmentation and apoptosis (29, 32).

We assessed cytochrome *c* release and caspase 9 activation in PAK-infected HeLa cells. As shown in Fig. 5A, cytochrome *c* release into cytoplasm occurred following infection by PAK or PAKexoST/exoS but not by PAKexoST/exoSE381A. Cleavage of procaspase 9 in infected HeLa cells showed the same strain dependence as that of cytochrome *c* release (Fig. 5B). Cleavage products of procaspase 9 resemble that seen upon staurosporine treatment of uninfected cells, which is known to trigger apoptosis through cytochrome *c* release and caspase 9 activation (29, 32). Curiously, the apparent molecular weights for the full-length procaspase 9 and its cleaved fragments were consistently higher by Western blotting in PAK-infected cells (Fig. 5B), suggesting a possible modification of caspase 9 resulting from bacterial infection. When HeLa cells were treated with a cell-permeable caspase 9-specific inhibitor before and during PAK infection, caspase 3 activity was reduced in a dose-dependent manner (Fig. 5C), indicating the direct and crucial involvement of the caspase 9 upstream of caspase 3 activation.

Because SP600125 was able to inhibit JNK phosphorylation and thereafter apoptosis induction (Fig. 3), we further assessed its impact on cytochrome *c* release and procaspase 9 processing. As shown in Fig. 6, both cytochrome *c* release (Fig. 6A) and cleavage of procaspase 9 (Fig. 6B) in PAK-infected cells

FIG. 4. Activation of c-Jun-mediated transcription is not required for ExoS-induced apoptosis. (A) HeLa cells were infected with PAK, PAK*exoST*/pUCP18*exoS* (ExoST/S), or PAK*exoST*/pUCP18*exoSE381A* (ExoST/EA) or left uninfected as control (lane C). At the indicated times p.i., cell lysates were blotted with antibody against phospho-c-Jun (at Ser63) and c-Jun. (B) HeLa cells were pretreated with 25 μ M CHX or left untreated (0 μ M CHX) for 2 h before being infected with PAK. At 5 h p.i., caspase 3 activities from HeLa cells were measured. Control samples (CTRL) are uninfected cells.

were inhibited by SP600125; densitometric analyses indicated that cytochrome *c* release and procaspase 9 processing is inhibited about 70% as a result of inhibiting JNK phosphorylation. These data strongly support the central role played by JNK in the ExoS-mediated apoptosis.

DISCUSSION

Despite the fact that ExoS induction of apoptosis is independent of de novo gene expression, the patterns of differentially altered gene expression observed in our study could be important signatures for the activation or stimulation of specific signal transduction pathways, such as JNK activation. An earlier report studying gene expression in the A549 lung pneumocyte cell line after exposure to *P. aeruginosa* identified host genes that are preferentially expressed upon infection by *P. aeruginosa* (39). Several of those genes are also seen in our present study, such as c-Jun in particular (data not shown).

One effect of ExoS ADPRT activity is to inhibit activation of cell survival pathways. The ExoS-dependent inhibition of ERK1/2 is consistent with previous work from other investigators (30). Although the specific contribution of p38 activation to survival in HeLa cells is unknown, p38 has been implicated in both apoptosis and anti-apoptosis signaling (31). Thus, inhibition of p38 phosphorylation by ExoS could have effects similar to those of ERK1/2 inhibition. In HeLa and other cell lines that are sensitive to PAK-induced apoptosis, inhibition per se of either ERK1/2 or p38 activation, alone or in combination, was not sufficient to induce apoptosis, as shown by the

studies here and elsewhere (41, 57). However, the cellular commitment to apoptosis is a response that represents the net integration of signals from cell survival and apoptotic signaling pathways. Thus, direct inhibition of any or all Ras-regulated survival pathways would increase the sensitivity of cells to apoptotic stimuli. In addition, the data presented here do not address the possible contributions to apoptosis induction by ExoS-triggered repression of antiapoptotic genes, which may represent a redundant mechanism of ExoS impairing Ras and/or other regulated survival signaling pathways.

The data here show that JNK1/2 action in response to ExoS ADPRT activity is a critical mediator of ExoS-triggered apoptosis. In addition, the apoptotic pathway appears to rely marginally on the changes of gene expression; instead, as a consequence of JNK phosphorylation, activation or modification of existing proteins, such as procaspases, appears to play a more significant role. This is supported by the following observations: (i) protein synthesis inhibitor does not block PAK-mediated apoptosis; (ii) *P. aeruginosa* strains that induce apoptosis in HeLa cells can activate JNK, whereas nonapoptotic strains failed to activate JNK; (iii) the activation of JNK in PAK-infected cells kinetically precedes and is required for cytochrome *c* release; (iv) inhibition of the JNK signaling represses apoptotic response in HeLa cells upon infection by PAK.

Activation of JNK following infection by an invasive *P. aeruginosa* strain was recently reported by Jendrosseck and co-workers (40), who had also demonstrated that upon infection of lung epithelial cells, *P. aeruginosa* induced an up-regulation

FIG. 5. ExoS of *P. aeruginosa* leads to cytochrome *c* release and caspase 9 activation. HeLa cells were infected with PAK, PAK*exoST*/pUCP18*exoS* (ExoST/S), or PAK*exoST*/pUCP18*exoSE381A* (ExoST/EA). (A) Immunoblot of cytochrome *c* content in the cytosol of infected cells. At the indicated times p.i., the cytosolic fractions were analyzed by immunoblot for cytochrome *c*. Lane C, uninfected HeLa control; lane P, cytochrome *c* positive control. (B) Immunoblot of full-length and cleaved caspase 9. The position of the full-length (procaspase 9) and cleaved products of caspase 9 are indicated. As positive controls for the apoptosis, uninfected HeLa cells were treated with either TNF- α -CHX (TNF) or 2 μ M staurosporine (STS). Lane C, uninfected control. (C) Caspase 3 activities measured at 5 h p.i. in uninfected (CTRL), PAK-infected (PAK), and staurosporine-treated (STS) cells. HeLa cells were pretreated with the indicated concentrations of caspase 9 inhibitor Z-LEHD-FMK or left untreated for 2 h prior to infection.

of CD95L/CD95 on the cell surface, leading to apoptosis in the lung epithelial cells (24). However, no specific factor from *P. aeruginosa* was identified in those studies. Our observations here and previous observations (41) specify that the type III toxin ExoS from an invasive strain can manipulate host cell signaling pathway to dictate their apoptotic outcome. Although CD95 (Apo-1 or Fas) expression was not shown to be preferentially up-regulated during the course of our microarray study (data not shown), the involvement of CD95L/CD95 could provide a clue for linking ExoS to the JNK activation, especially at the posttranscription level. In addition, a more recent study by Coopersmith and colleagues (8) reported that overexpression of Bcl-2 effectively inhibited gut epithelial apoptosis and gave mice a survival advantage in *P. aeruginosa* pneumonia-induced sepsis. These observations correlate with our results implicating the involvement of cytochrome *c*.

Apoptosis can be either advantageous or detrimental to the host throughout the course of a respiratory infection (4). Generally, in infections involving intracellular pathogens, such as *Chlamydia* species, apoptosis of infected host defense and epithelial cells favors clearance of bacterial pathogens (15, 47). However, for extracellular pathogens, as in the case of *Burk-*

FIG. 6. JNK inhibitor SP600125 blocks cytochrome *c* release and procaspase 9 cleavage in PAK-infected HeLa cells. HeLa cells were pretreated with 25 or 50 μ M SP600125 and infected with wild-type PAK. Cell extracts were isolated at 4 h p.i. and analyzed by Western blotting for cytochrome *c* (A) or caspase 9 (B); only lysates treated with 25 μ M SP600125 are shown.

Proposed pathways for the ExoS mediated apoptosis

FIG. 7. Proposed pathways for the ExoS-mediated apoptosis. ExoS is delivered into host cells through type III machinery. Both proapoptotic and antiapoptotic (or proliferation) signaling pathways are implicated. In the proapoptotic pathway, ExoS targets unknown intracellular factor(s) to initiate the apoptotic signal, leading to the activation of JNK1, which results in cytochrome *c* release and caspase 9 activation. Caspase 3 is then activated and executes the apoptosis program. This pathway is also potentiated by inhibition of an antiapoptotic pathway by the ExoS, which likely occurs through the inhibition of Ras and Ras-associated Raf-MEK-ERK signaling.

holderia cepacia, apoptosis of host inflammatory cells may be advantageous to the pathogens (4, 37). In particular, apoptosis of alveolar epithelial cells upon infection by *Streptococcus pneumoniae* appears detrimental to the host, as this contributes to impaired lung function (25), suggesting a possible mechanism for lung tissue degeneration in *P. aeruginosa*-infected patients. However, the matter could become more complicated—and sometimes controversial—in *P. aeruginosa* infections due to their differences in terms of site, type, and even progression of bacterial infection. While activation of the CD95/CD95 ligand system in lung epithelial cells may protect the host from *P. aeruginosa* infection by targeting of *P. aeruginosa* into apoptotic bodies or by CD95-triggered secretion of defensins and/or cytokines into bronchi, where they may then kill extracellular bacteria (24, 34), inhibiting gut epithelial apoptosis was associated with a survival advantage in *P. aeruginosa* pneumonia-induced sepsis in another murine model, possibly resulting from restored gut barrier function (8). Taken together, successful control of respiratory infections requires that the host maintain the right balance between apoptotic and antiapoptotic pathways, as failure to do so may convert an acute self-limiting infection into a chronic disabling disease.

Based on our observation, we propose a model by which the proapoptotic and antiapoptotic signaling pathways are modu-

lated in HeLa cells after infection by invasive *P. aeruginosa*. As shown in Fig. 7, antiapoptotic signaling in HeLa cells is likely to be suppressed due to inactivation of Ras by ExoS, sensitizing the host cells to apoptotic signaling. Simultaneously and more dominantly, the ADPRT domain of ExoS modifies target(s) in HeLa cells, leading to JNK1 activation (Fig. 7). The activated JNK1 promotes the release of cytochrome *c*, which is followed by activation of caspase 9 and the executive caspase 3. In this model, potential targets of JNK that may regulate cytochrome *c* release include members of the Bcl-2 group of apoptosis regulatory proteins. Repressing antiapoptotic proteins Bcl-XL and Bcl-2, and/or activating pro-apoptotic protein such as Bid can lead to cytochrome *c* release following cellular stress (42, 48, 56). The schematic signal transduction (Fig. 7) highlights the involvement of JNK1/2 activation in parallel to interference of Ras signaling of cell survival pathways. However, there are a number of intriguing questions which need to be answered to completely illustrate the detailed mechanisms and regulatory checkpoints. These include (i) the extent of the role played by ExoS-mediated inhibition of the Ras-Raf-MEK-ERK signaling pathway in the overall commitment of cells to apoptosis; (ii) the steps downstream of JNK activation that lead to cytochrome *c* release; (iii) the intermediates following introduction of ExoS that propagate the signal leading to JNK1/2 activation; and finally (iv) how the signaling linkage between ExoS and activated JNK1/2 is regulated to determine the fate of cells infected by PAK as well as disease development within the host.

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