Requirement of p21-activated Kinase (PAK) for *Salmonella typhimurium*–induced Nuclear Responses

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Summary

Salmonella typhimurium has sustained a long-standing association with its host and therefore has evolved sophisticated strategies to multiply and survive within this environment. Central to *Salmonella* pathogenesis is the function of a dedicated type III secretion system that delivers bacterial effector proteins into the host cell cytoplasm. These effectors stimulate nuclear responses and actin cytoskeleton reorganization leading to the production of proinflammatory cytokines and bacterial internalization. The stimulation of these responses requires the function of Cdc42, a member of the Rho family of small molecular weight GTPases, and SopE, a bacterial effector protein that stimulates guanine nucleotide exchange on Rho GTPases. However, nothing is known about the role of Cdc42 effector proteins in *S. typhimurium*–induced responses. We showed here that *S. typhimurium* infection of cultured epithelial cells results in the activation of p21-activated kinase (PAK), a serine/threonine kinase that is an effector of Cdc42-dependent responses. Transient expression of a kinase-defective PAK blocked both *S. typhimurium*– and SopE-induced c-Jun NH_2-terminal kinase (JNK) activation but did not interfere with bacteria-induced actin cytoskeleton rearrangements. Similarly, expression of SH3-binding mutants of PAK did not block actin-mediated *S. typhimurium* entry into cultured cells. However, expression of an effector loop mutant of Cdc42Hs ($Cdc42Hs^{C40}$) unable to bind PAK and other CRIB (for Cdc42/Rac interacting binding)-containing target proteins resulted in abrogation of both *S. typhimurium*–induced nuclear and cytoskeletal responses. These results show that PAK kinase activity is required for bacteria-induced nuclear responses but it is not required for cytoskeletal rearrangements, indicating that *S. typhimurium* stimulates cellular responses through different Cdc42 downstream effector activities. In addition, these results demonstrate that the effector loop of Cdc42 implicated in the binding of PAK and other CRIB-containing target proteins is required for both responses.

Key words: Cdc42 • signal transduction • actin cytoskeleton • bacterial pathogenesis

Bacterial pathogens that have sustained a long-standing association with their hosts have evolved sophisticated strategies to multiply and survive within them (1). These strategies often involve a very sophisticated subversion of basic host cellular functions. An example of this type of highly adapted pathogen is *Salmonella enterica*, an enteropathogenic bacteria that can cause a wide range of infectious diseases in humans and other animals (2).

A central element in the pathogenesis of *S. enterica* is the function of a specialized protein secretion and translocation system often referred to as type III or contact dependent (3). The specific function of this system is the delivery of bacterial effector proteins into the host cell cytoplasm to stimulate or interfere with cellular responses. One of the earliest cellular responses observed after *Salmonella* infection is pronounced membrane ruffling and actin cytoskeleton rearrangements at the point of bacteria–host cell contact (4–6). These responses, which are accompanied by profuse macropinocytosis, ultimately direct the internalization of the bacteria, a process that is essential for pathogenicity. Another cellular response stimulated by the translocated bacterial effectors is the activation of the mitogen-activated protein (MAP) kinases extracellular signal regulatory kinase (ERK), c-Jun NH_2-terminal kinase (JNK),[1] and p38 (7). Stimulation of these MAP kinase pathways leads to the activation of the transcription factors AP-1 and nuclear factor

[1]*Abbreviations used in this paper:* CRIB, Cdc42/Rac interacting binding motif; GFP, green fluorescent protein; JNK, c-Jun NH_2-terminal kinase; moi, multiplicity of infection; PAK, p21-activated kinase.

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(NF)-κB, resulting in the production of proinflammatory cytokines such as IL-8. This response is important for the establishment of the inflammatory diarrhea that ensues upon *Salmonella* infection.

Previous studies from our laboratory have implicated the small molecular weight GTP-binding proteins Cdc42 and Rac-1 in the cellular responses stimulated by *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) (8). Expression of dominant interfering mutants of Cdc42Hs ($Cdc42Hs^{N17}$) and to a lesser extent Rac-1 ($Rac\text{-}1^{N17}$) abolished bacteria-induced actin cytoskeleton rearrangements, macropinocytosis, and subsequent bacterial internalization into cultured cells. Furthermore, $Cdc42Hs^{N17}$ also abolished the nuclear responses stimulated by *S. typhimurium*, indicating that this GTPase is required for both morphological and transcriptional responses induced by these bacteria. More recent studies from our laboratory have identified a bacterial effector protein from *S. typhimurium,* termed SopE, that upon delivery through the bacterial type III protein translocation system or when microinjected or transiently expressed in cultured cells is capable of stimulating both membrane ruffling and JNK activation (9). The stimulation of these responses by SopE was shown to require Cdc42 and Rac-1. Consistent with this finding, SopE was found to bind the nucleotide-free forms of Cdc42 and Rac-1 and to stimulate guanine nucleotide exchange on these GTPases. Thus, *S. typhimurium* stimulates cellular responses by delivering an activator of Rho GTPases to the cell cytosol using a specialized protein secretion and delivery system.

Although a considerable amount of work by many laboratories has implicated Cdc42 and Rac in the regulation of a variety of cellular processes, such as the organization of the actin cytoskeleton and focal adhesion, cytokinesis, and the stimulation of nuclear and mitogenic responses, the actual mechanisms by which these Rho GTPases regulate cellular activities remain poorly understood (10–15). Several proteins have been identified that directly interact with the activated (GTP-bound) forms of these GTPases and therefore are likely candidates to be effectors of cellular responses involving these small G proteins (16–23). Some of these putative effector proteins have kinase activity or have domain structures that suggest their involvement in the regulation of signaling pathways or the modulation of the actin cytoskeleton. Many but not all of the Cdc42 and Rac-1 binding proteins exhibit a conserved 16–amino acid motif termed CRIB (for Cdc42/Rac interacting binding) or p21-binding domain (PBD) that is involved in the binding of these putative effectors to a specific effector loop of these GTPases (24). Among this subset of targets of Rho GTPases are a group of highly related serine/threonine kinases known as p21-activated kinases (PAKs [25]). At least three members of this family (PAK1 or PAKα, PAK2 or PAKγ, and PAK3 or PAKβ) have been identified in mammalian tissues. PAKs have been implicated in a variety of cellular processes, such as the organization of the actin cytoskeleton and focal adhesion complexes and the stimulation of stress kinases such as JNK and p38 (26–28). In addition, this protein family has also been implicated in the pathogenesis of HIV infections by interacting with the viral protein Nef (29, 30).

Further advances in the understanding of the function of the Rho family of small G proteins have come from the availability of effector domain mutations in these small GTPases that are differentially impaired in downstream signaling pathways. This approach has allowed the identification of effector loops of Rho GTPases specifically involved in actin remodeling, transcriptional activation, transformation, cell cycle progression, and the coordination of the cross-talk between different Rho GTPases (31–34).

Although studies have provided major insight into the mechanisms by which *S. typhimurium* stimulates host cell responses by activating Rho GTPases, nothing is known about the potential involvement of direct targets of these small G proteins in the cellular responses stimulated by this bacterium. It is also unknown whether the nuclear and cytoskeletal responses stimulated by *S. typhimurium* are mediated by the same or different effectors of Rho GTPases. In this report, we describe the involvement of the Cdc42 and Rac-1 effector protein PAK in the *S. typhimurium*–induced cellular responses. We found that *S. typhimurium* infection of cultured cells results in the activation of PAK. The kinase activity of this effector protein was found to be required for the bacteria-induced nuclear responses but was not required for the actin cytoskeleton–mediated *S. typhimurium* entry into host cells. Furthermore, we show that expression of an effector domain loop mutant of Cdc42Hs ($Cdc42Hs^{C40}$) unable to bind CRIB domain–containing proteins blocked both actin cytoskeleton and nuclear responses induced by *S. typhimurium*. Therefore, these studies show that *S. typhimurium* uses different Cdc42 downstream effector activities to modulate host cellular responses.

Materials and Methods

Bacterial Strains and Plasmids. The wild-type *S. typhimurium* strain SL1344 and its isogenic mutant derivative strain SB161, which carries a nonpolar mutation in the *invG* gene, have been described previously (35). Plasmid J3HmPAK-3 encoding HA epitope–tagged mPAK-3 has been described previously (27). Plasmid pSB961 was constructed by subcloning the 1.7-kb BamHI fragment from J3HmPAK-3KR plasmid into the EcoRI site of pSB936. The resulting plasmid encodes the mPAK-3 kinase–defective mutant at the first cistron and the green fluorescent protein (GFP) at the second cistron. Plasmids pSB969, pSB970, pSB971, and pSB972 were constructed by subcloning the 1.7-kb BamHI fragments from pGEM-P1PAK (encoding PAK^{A12A14}), pGEM-P2PAK (encoding PAK^{A36A39}), pGEM-P3PAK (encoding $PAK^{A165A168}$), and pGEM-P4PAK (encoding $PAK^{A213A216}$), respectively, into the EcoRV site of the dicistronic expression vector pSB965. The resulting plasmids encode the different PAK mutants at the first cistron and GFP at the second cistron. Plasmid pSB974, which encodes $Cdc42Hs^{C40}$, was constructed by introducing a point mutation (codon 40 of Cdc42Hs changed from TAT to TGT) into the coding sequence of wild-type Cdc42Hs encoded by plasmid pSB944. The resulting plasmid encodes $Cdc42Hs^{C40}$ at the first cistron and GFP at the second cistron.

Cell Transfection and Immunofluorescence Microscopy. COS-1 cells were grown to subconfluence on glass coverslips placed in 24-well

culture dishes and transfected by the calcium phosphate method (36) using a total of 1 μg of DNA per well. For PAK localization studies, COS cells were infected with wild-type *S. typhimurium* with a multiplicity of infection (moi) of 20. At different times after infection, cells were fixed in 3.7% formaldehyde in PBS for 1 h, permeabilized in the presence of 0.15% Triton X-100 for 5 min, incubated for 1 h in blocking buffer (PBS, 5% milk), and stained as described above using a rabbit polyclonal antibody that recognizes all isoforms of PAK (Santa Cruz Biotech, Inc.). Rhodamine-conjugated phalloidin (1 U/ml in PBS; Molecular Probes) was used to visualize the actin cytoskeleton, and 4′,6′-diamidino-2-phenylindole (DAPI) to stain DNA. Coverslips were mounted onto slides with Vectashield mounting solution (Vector Labs, Inc.) and visualized under a 40× objective in a Nikon Diaphot fluorescence microscope. Images were captured with a Hamamatsu 75i CCD camera and pseudocolored using an Argus 20 image processor.

Bacterial Internalization Assay. Bacterial internalization was measured as described elsewhere (8). In brief, COS-1 cells grown on glass coverslips were transfected with a total of 1 μg of DNA of dicistronic vectors expressing different forms of PAK or Cdc42Hs in the first cistron and GFP in the second cistron. 48 h after transfection, the cells were washed and infected at an moi of 40 with wild-type *S. typhimurium.* After 1 h of infection, cells were washed and internalized bacteria were detected using a staining protocol that allows the distinction between extracellular and intracellular bacteria (8). Cells expressing the different PAK or Cdc42Hs constructs were identified by the coupled expression of GFP.

JNK and PAK Protein Kinase Assays. COS-1 cells were grown in 6-cm tissue culture dishes and transfected by the calcium phosphate method using a total of 10 μg of DNA. When appropriate, 48 h after transfection cells were infected with wild-type *S. typhimurium* or the isogenic *invG* mutant strain SB161 with an moi of 20. At different times after infection, cells were lysed in lysis buffer (1% NP-40, 40 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM sodium vanadate), and the levels of JNK or PAK activity were measured by an immunocomplex kinase assay as described elsewhere (37). The relative amounts of substrate phosphorylation were quantitated with a PhosphorImager (Storm; Molecular Dynamics). Readings were standardized relative to a given sample that was assigned the value 1. The levels of Flag-JNK-1, HA-PAK, or M45-SopE in cell lysates were determined by immunoblotting with the respective antibodies.

Results

S. typhimurium Induces the Activation of PAK in a Type III Secretion–dependent Manner. To gain insight into the role of downstream effectors of Cdc42 in the *S. typhimurium*–induced cytoskeletal and nuclear responses, we investigated whether *S. typhimurium* infection of cultured cells would result in PAK activation. COS-1 cells were transfected with HA epitope–tagged mPAK-3, a ubiquitously distributed isoform of the PAK family of protein kinases. Transfected cells were then infected with either wild-type *S. typhimurium* or an isogenic derivative strain carrying a null mutation in *invG.* InvG is an essential component of the type III secretion apparatus, and therefore failure to express this protein results in a strain that is unable to induce cellular responses dependent on this system. At different times after infection, the PAK activity in infected cells was measured in an immunocomplex kinase assay as described in Materials and Methods. As shown in Fig. 1, wild-type *S. typhimurium* induced significant activation of PAK. PAK activation was observed as early as 5 min after infection, reaching a maximum at ~10 min after infection and rapidly decreasing over time. In contrast, the signaling-defective *S. typhimurium invG* mutant strain failed to induce PAK activation even after 60 min of infection. These results indicate that *S. typhimurium* interaction with host cells results in the activation of PAK, and such activation is strictly dependent on the function of the signaling-associated type III secretion system.

We then tested whether *S. typhimurium* infection of cultured cells resulted in a redistribution of endogenous PAK. It has been previously shown that recruitment of PAK to the cell membrane results in its activation (38). COS cells were infected with wild-type *S. typhimurium* for various periods of time, then fixed and stained with a polyclonal anti-PAK antibody and rhodamine-labeled phalloidin to visualize the actin cytoskeleton. As shown in Fig. 2, A and B, *S. typhimurium* infection resulted in the rapid recruitment of PAK to the bacterial-stimulated membrane ruffles. Ruffles stained by the PAK antibodies were seen as early as 5 min after infection. Interestingly, such a recruitment was seen in only a subset of the membrane ruffles stimulated by *S. typhimurium.* In fact, the recruitment of PAK to the membrane ruffles appears to be transient, as later (30 min) in infection the proportion of ruffles exhibiting PAK staining significantly decreased. The recruitment of PAK to only a subset of agonist-induced membrane ruffles has been previously

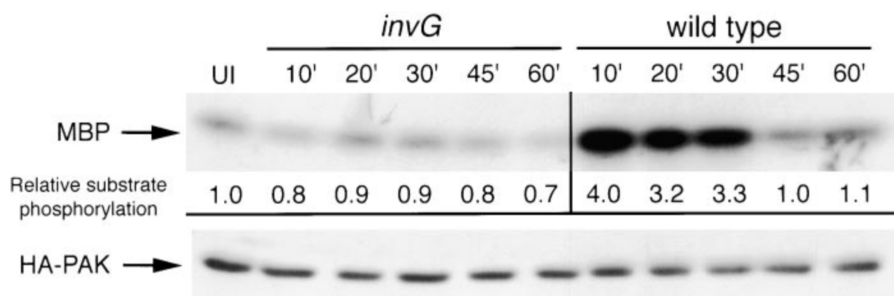

Figure 1. *S. typhimurium* activates PAK. COS-1 cells were transfected with a vector encoding HA-tagged PAK and subsequently infected with either wild-type *S. typhimurium* or the noninvasive *invG* mutant derivative. PAK activity was measured at the indicated times after infection in an immunocomplex kinase assay. Substrate phosphorylation was measured with a PhosphorImager, and phosphorylation levels of all samples were standardized relative to the level of uninfected cells, which was assigned the value 1. Expression levels of HA-PAK in the different lysates were determined by Western immunoblot analyses with an mAb directed to the HA epitope tag. UI, uninfected control.

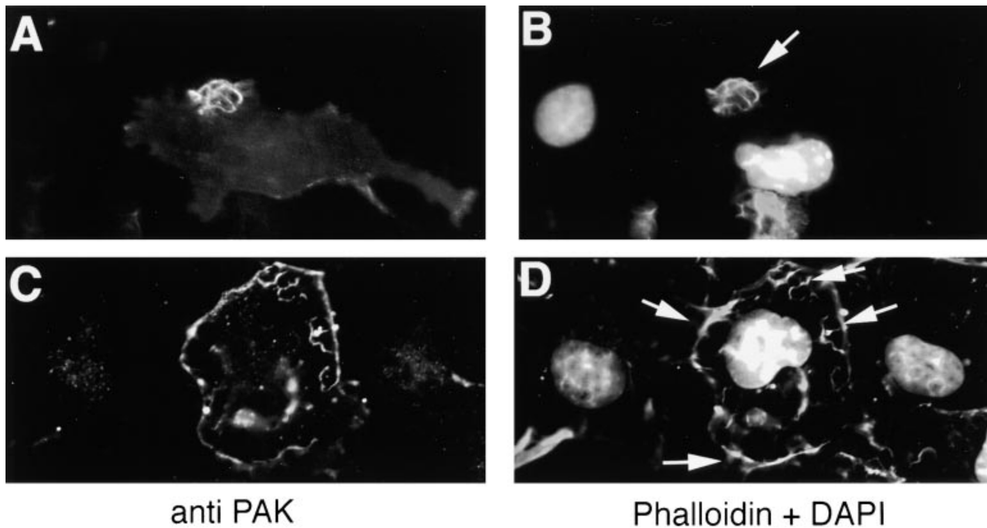

Figure 2. Recruitment of PAK to the *S. typhimurium*– and SopE-stimulated membrane ruffles. (A and B) COS cells were infected with wild-type *S. typhimurium*, and cells were stained with an antibody directed to PAK (A) and rhodamine-labeled phalloidin and DAPI to visualize actin cytoskeleton rearrangements and DNA (B). Alternatively, COS-1 cells were transfected with a vector expressing the bacterial effector SopE (C and D), and cells were stained with an antibody directed to PAK (C) and rhodamine-labeled phalloidin and DAPI to visualize actin cytoskeleton rearrangements and DNA (D). Arrows point at membrane ruffles.

reported (39). Infection of COS cells with the *invG* mutant strain did not result in any detectable change in the localization of endogenous PAK (data not shown). Taken together, these results indicate that *S. typhimurium* is capable of changing the distribution of PAK in infected cells through the function of its signaling-associated type III protein secretion and translocation system.

SopE is an effector protein delivered by *S. typhimurium* into host cells via its type III secretion system (40). We have shown previously that transient expression of this protein results in membrane ruffling and JNK activation as a result of the direct stimulation of Cdc42 and Rac-1 by SopE (9). Therefore, we examined the distribution of PAK in COS cells transiently expressing the bacterial effector SopE. As shown in Fig. 2, C and D, PAK was also recruited to the SopE-stimulated membrane ruffles.

Expression of a Kinase-defective PAK^{R297} Mutant Blocks S. typhimurium– and SopE-induced JNK Activation. We have previously shown that *S. typhimurium* stimulates the stress-activated protein kinase JNK in a Cdc42-dependent manner (8). The PAK family of proteins has been implicated in the Cdc42- and Rac-1–mediated activation of both JNK and p38 protein kinases. The finding that *S. typhimurium* infection of host cells leads to the activation of PAK prompted us to examine the role of this kinase in *S. typhimurium*–induced JNK activation. COS-1 cells were cotransfected with a vector encoding Flag epitope–tagged JNK-1 and a vector encoding either wild-type PAK, the kinase-defective PAK^{R297} mutant, or the empty vector control. Transfected cells were infected with wild-type *S. typhimurium*, and the activity of JNK was measured in an immunocomplex kinase assay as described in Materials and Methods. As shown in Fig. 3 A, expression of the kinase-defective PAK^{R297} mutant blocked *S. typhimurium*–induced JNK activation. Expression of wild-type PAK did not result in significant inhibition of bacteria-induced JNK activation (data not shown). Since expression of PAK^{R297} did not result in inhibition of other Cdc42-dependent events

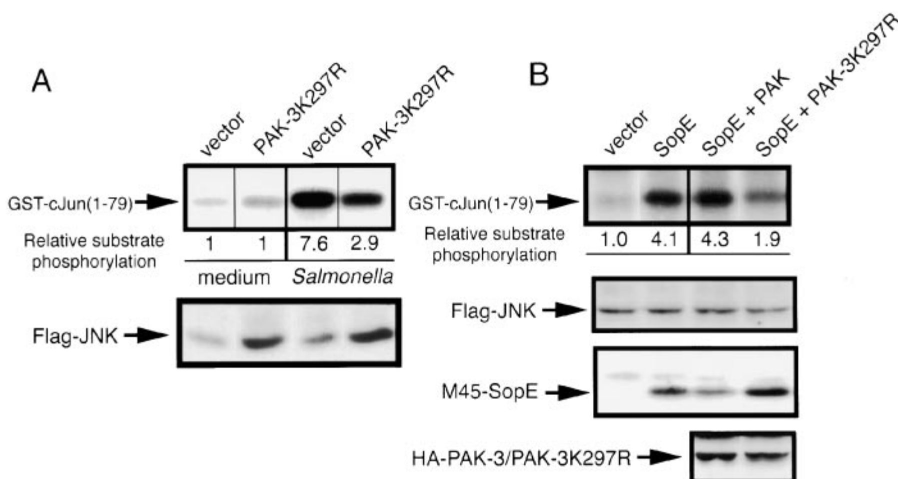

Figure 3. Kinase-defective PAK blocks *S. typhimurium*– and SopE-induced JNK activation. (A) COS-1 cells were cotransfected with a vector encoding a Flag epitope–tagged JNK-1 and a 2.5× excess of a plasmid encoding the kinase-defective PAK^{R297} or the vector alone. Cells were subsequently infected with *S. typhimurium* for 30 min or mock treated with infection medium for the same amount of time, and the JNK activity was measured in an immunocomplex kinase assay. The relative substrate phosphorylation was determined with a PhosphorImager, and phosphorylation levels of all samples were standardized relative to the level of mock-treated cells, which were assigned the value 1. The expression levels of Flag-JNK in the different lysates was determined by Western immunoblot analyses with an mAb directed to the Flag epitope. (B) COS-1 cells were cotransfected with a vector encoding a Flag epitope–tagged JNK-1 along with vectors encoding SopE and a plasmid encoding either PAK, the kinase-defective mutant PAK-3^{K297R}, or the vector alone. JNK kinase activity and the levels of the different proteins in cell lysates were determined as indicated in A.

(see below), the inhibitory effect of this mutant cannot be explained by nonspecific sequestration of Cdc42. Therefore, these results indicate that PAK is required for *S. typhimurium*–induced nuclear responses.

We also tested whether the activation of JNK induced by the bacterial effector SopE (40) was also dependent on the function of PAK. COS-1 cells were transfected with a vector encoding Flag epitope–tagged JNK-1, the $SopE_{78–240}$ effector protein along with wild-type PAK, the kinase-defective mutant PAK^{R297}, or the empty vector control. Cotransfection of SopE with the kinase-defective PAK^{R297} mutant effectively blocked JNK activation (Fig. 3 B). In contrast, cotransfection of $SopE_{78–240}$ with wild-type PAK did not result in significant inhibition of SopE-mediated JNK activation. Taken together, these results implicate PAK in the nuclear responses stimulated by wild-type *S. typhimurium* and its effector protein SopE.

Expression of Kinase-defective and SH3-binding Mutants of PAK Does Not Block S. typhimurium Entry into Cultured Cells. In addition to the stimulation of the stress-activated kinases JNK and p38, the PAK family of protein kinases has been implicated in the organization of the actin cytoskeleton (26, 28). Furthermore, we showed that PAK is transiently recruited to the *S. typhimurium*– and SopE-induced membrane ruffles (Fig. 2). Therefore, we investigated the role of the kinase activity of this effector molecule in the actin cytoskeleton–mediated *S. typhimurium* internalization into cultured cells. A kinase-defective PAK mutant (PAK^{R297}) was expressed in COS-1 cells using a dicistronic expression system in which the cells expressing PAK^{R297} could be identified by the coupled expression of GFP. Transfected cells were infected with wild-type *S. typhimurium*, and bacterial internalization was quantified by a staining protocol that distinguishes extracellular and intracellular bacteria, as described in Materials and Methods. As shown in Fig. 4, expression of a kinase-defective PAK did not inhibit bacterial entry into host cells. As previously shown, expression of dominant-negative Cdc42Hs ($Cdc42Hs^{N17}$) effectively blocked bacterial internalization. These results indicate that the kinase activity of PAK is not required for actin cytoskeleton–mediated *S. typhimurium* internalization into host cells. Since this activity is required for bacteria-induced JNK activation (see above), these results also show that the *S. typhimurium* stimulation of actin cytoskeleton reorganization and nuclear responses are mediated by different downstream effector activities of Cdc42 signaling.

In addition to the conserved kinase domain, the PAK family of proteins exhibits other highly conserved structural features, such as the presence at its NH_2 terminus of several proline-rich regions resembling SH3-binding domains (27). At least one of these domains has been implicated in regulating the formation of polarized membrane ruffles and focal complexes and in the binding of PAK to the SH3-containing adapter protein Nck (41). To examine the potential involvement of the NH_2-terminal proline-rich regions of PAK in *S. typhimurium* internalization into host cells, we transiently expressed in COS-1 cells mutants of PAK (PAK^{A12A14}, PAK^{A36A39}, $PAK^{A165A168}$, and $PAK^{A213A216}$) containing changes in the conserved proline-rich NH_2-terminal domains. Although not formally investigated, we made the assumption that if any of these domains were required for *S. typhimurium*–induced cytoskeletal responses, transient expression of these mutants might result in a dominant-negative effect. Western blot analysis showed that all mutant forms of PAK were expressed in the transfected cells (data not shown). Transfected cells were infected with wild-type *S. typhimurium*, and the number of internalized bacteria in cells expressing the different mutant PAKs was determined as described in Materials and Methods. As shown in Fig. 4, bacterial internalization was not affected by the expression of any of the PAK mutants tested. These results suggest that the SH3-binding domains of PAK may not be required for actin cytoskeleton–mediated *S. typhimurium* internalization into host cells. However, the presence of multiple SH3-binding domains may prevent observation of the potential dominant-negative effect resulting from the expression of single SH3-binding domain mutants.

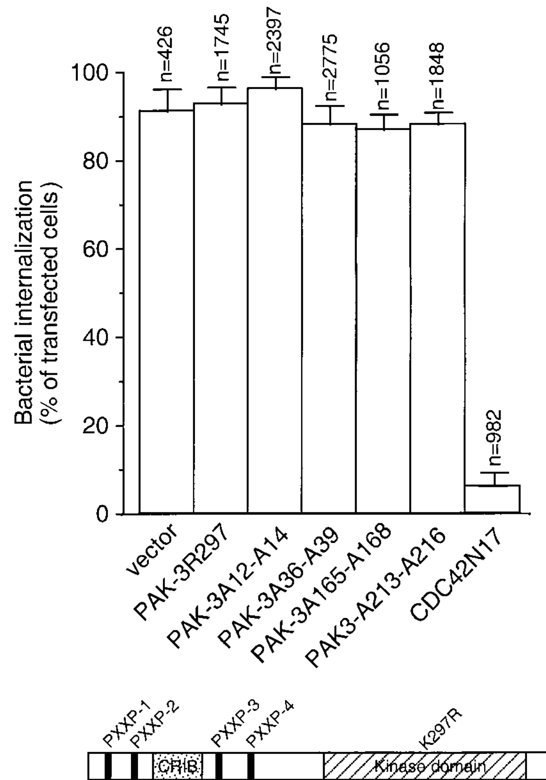

Figure 4. Effect of the expression of PAK mutants on *S. typhimurium* internalization into host cells. COS-1 cells were transfected with double cistronic vectors (pSB961, pSB969, pSB970, pSB971, pSB972) that direct the coupled expression of different PAK mutants and GFP as indicated. Transfected cells were infected with *S. typhimurium* and subsequently examined for the presence of internalized bacteria as indicated in Materials and Methods. Bacterial invasion is indicated as the percentage of transfected cells that exhibited three or more internalized bacteria.

Expression of $Cdc42Hs^{C40}$ Blocks Both Cytoskeletal and Nuclear Responses Induced by S. typhimurium. In addition to PAK, Cdc42 binds to several putative effector proteins that contain a conserved 16–amino acid domain, termed CRIB or p21-binding domain (PBD) (24). Effector domain muta-

tion analysis of Cdc42 has identified a critical residue for binding to this domain. Thus, Cdc42 carrying a Y to C mutation at residue 40 was unable to bind to all CRIB domain–containing proteins tested, including PAK, Wiskott-Aldrich syndrome protein (WASP), MSE55, and the *Caenorhabditis elegans* protein F09F7 (32). Since this effector loop mutant is unable to bind CRIB-containing effector proteins, we reasoned that if any of these effectors were required for *S. typhimurium*–induced responses, such a mutant should act as dominant interfering by nonproductively binding the bacterial effector SopE and thereby effectively titrating it out. Therefore, to investigate the potential role of CRIB domain–containing Cdc42 effector proteins in *S. typhimurium*–stimulated cellular responses, we transiently expressed in COS-1 cells the effector domain binding mutant $Cdc42Hs^{C40}$. We first examined the effect of expression of $Cdc42Hs^{C40}$ in *S. typhimurium*–induced JNK activation. COS-1 cells were cotransfected with a vector encoding Flag epitope–tagged JNK-1 and a vector encoding $Cdc42Hs^{C40}$, $Cdc42Hs^{N17}$, or the empty vector control. Transfected cells were infected with wild-type *S. typhimurium*, and the activity of JNK was measured in an immunocomplex kinase assay. As shown in Fig. 5 A, the expression of $Cdc42Hs^{C40}$ effectively blocked *S. typhimurium*–induced JNK activation. The inhibitory effect of $Cdc42Hs^{C40}$ was comparable to that of $Cdc42Hs^{N17}$. In contrast, transfection of wild-type Cdc42Hs or the empty vector control did not result in any measurable inhibition of bacteria-induced JNK activation (Fig. 5 A). These results indicate that a Cdc42 effector protein(s) that binds to the CRIB-binding domain of Cdc42 is required for *S. typhimurium*–induced JNK activation. Expression of $Cdc42Hs^{C40}$ also blocked *S. typhimurium*–induced PAK activation, which is consistent with the involvement of this effector in bacteria-induced nuclear responses (Fig. 5 B). The inhibiting effect of $Cdc42Hs^{C40}$ was equivalent to that of $Cdc42Hs^{N17}$. These results also demonstrate that $Cdc42Hs^{C40}$ can effectively exert a dominant interfering effect on *S. typhimurium*–induced signaling.

We then tested the effect of the expression of $Cdc42Hs^{C40}$ on the actin cytoskeleton reorganization and membrane ruffling induced by *S. typhimurium* or the transient expression of its effector SopE. COS-1 cells were transfected with a double cistronic vector expressing $Cdc42Hs^{C40}$ and GFP or the empty vector control. Transfected cells were then infected with wild-type *S. typhimurium*, and the actin cytoskeleton rearrangements resulting from bacterial infection were examined by rhodamine-phalloidin staining. Alternatively, internalized bacteria were enumerated as described in Materials and Methods. Expression of $Cdc42Hs^{C40}$ effectively prevented both *S. typhimurium*–induced actin cytoskeleton rearrangements (Fig. 6 A) and bacterial internalization (Fig. 6 B). Similarly, expression of $Cdc42Hs^{C40}$ also blocked the cytoskeletal rearrangements induced by the transient expression of SopE (Fig. 6 C). In contrast, expression of the constitutively active effector loop mutant $Cdc42Hs^{L61C40}$ did not inhibit bacterial internalization (Fig. 6 B). $Cdc42Hs^{L61}$ does not efficiently bind the bacterial effector SopE (9); therefore, introduction of the activating mutation relieves the dominant-negative effect conferred by the C40 effector loop mutation, since this mutant is unable to sequester the bacterial effector. Taken together, these results indicate that a CRIB domain–containing effector protein(s) (such as PAK) or another effector protein(s) that binds to the same effector loop of Cdc42 is required for bacterial internalization as well as *S. typhimurium*– and SopE-induced actin cytoskeleton reorganization and nuclear responses.

Discussion

S. typhimurium induces nuclear and morphological responses in infected cells in a manner that is absolutely dependent on the function of the small GTP-binding protein Cdc42 (8). The related GTPase Rac-1 also plays a significant but clearly less important role in these responses. It is now apparent that *S. typhimurium* triggers these cellular responses by delivering into the host cell cytosol at least one bacterial effector protein that directly stimulates GDP/GTP nucleotide exchange on these Rho GTPases (9). The delivery of the effector proteins is carried out by a complex

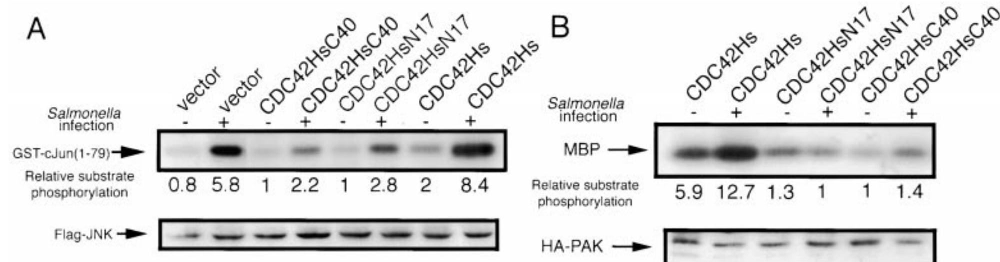

Figure 5. Expression of $Cdc42Hs^{C40}$ blocks *S. typhimurium*–induced JNK and PAK activation. (A) COS-1 cells were cotransfected with a vector encoding a Flag epitope–tagged JNK-1 and a 2.5× excess of a plasmid encoding Cdc42Hs, $Cdc42Hs^{C40}$ (defective in binding CRIB domain–containing proteins), $Cdc42Hs^{N17}$ (dominant negative), or the vector alone. Cells were subsequently infected with *S. typhimurium* for 30 min or mock treated with infection medium for the same amount of time, and the JNK activity was measured in an immunocomplex kinase assay. The relative substrate phosphorylation was determined with a PhosphorImager, and values were standardized relative to each others' levels. The expression levels of Flag-JNK in the different lysates were determined by Western immunoblot analyses with an mAb directed to the Flag epitope. (B) COS-1 cells were cotransfected with a vector encoding an HA epitope–tagged PAK and a 2.5× excess of a plasmid encoding Cdc42Hs, the dominant-negative mutant $Cdc42Hs^{N17}$, the effector loop mutant $Cdc42Hs^{C40}$, or the vector alone. Cells were subsequently infected with *S. typhimurium* for 20 min or mock treated with infection medium for the same amount of time, and the PAK activity was measured in an immunocomplex kinase assay. The relative substrate phosphorylation was determined with a PhosphorImager as indicated in A. The expression levels of HA-PAK in the different lysates were determined by Western immunoblot analyses with an mAb directed to the HA epitope.

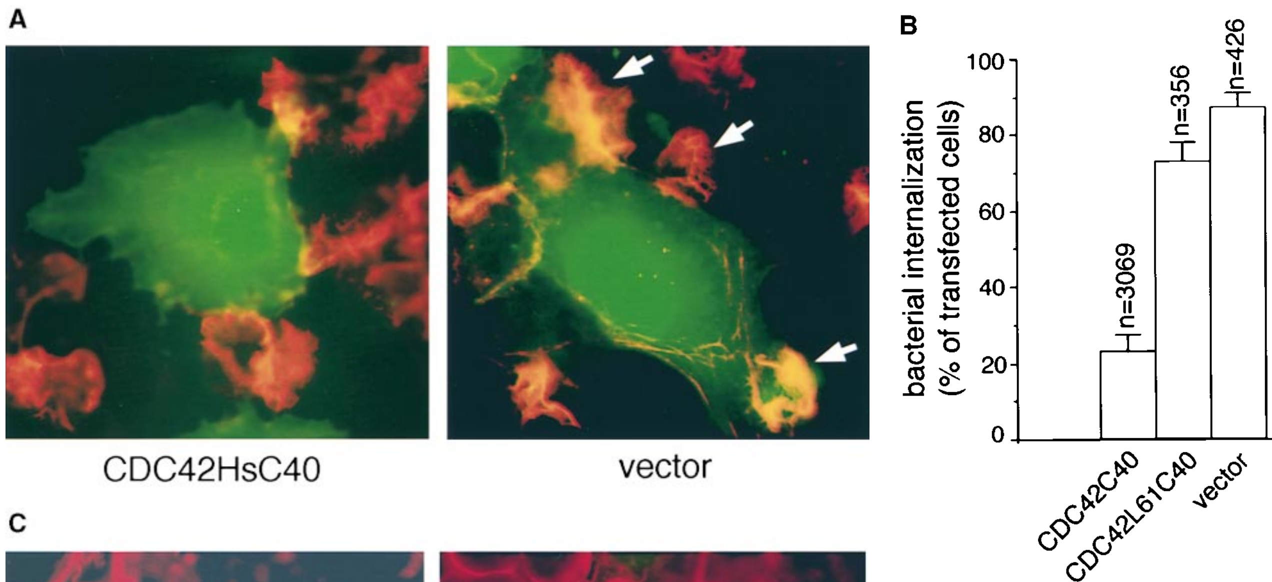

Figure 6. Expression of Cdc42Hs^{C40} blocks *S. typhimurium*– and SopE-induced cytoskeleton rearrangements and bacterial internalization. (A) COS-1 cells were transfected with a double cistronic vector that directs the coupled expression of Cdc42Hs^{C40} and GFP, or the empty vector control expressing GFP alone. Transfected cells were infected with *S. typhimurium* and subsequently stained with rhodamine-phalloidin to visualize the actin cytoskeleton. Note the absence of membrane ruffles in cells transfected with Cdc42Hs^{C40} (green cell, left panel) and abundant ruffles in cells transfected with the empty vector (arrows on green cell, right panel). (B) Transfected cells expressing Cdc42Hs^{C40}, Cdc42Hs^{L61C40}, or the vector alone were examined for the presence of internalized bacteria as indicated in Materials and Methods. Bacterial invasion is indicated as the percentage of transfected cells that exhibited three or more internalized bacteria. (C) COS-1 cells were cotransfected with a double cistronic vector that directs the coupled expression of Cdc42Hs^{C40} and GFP or the empty vector control (expressing only GFP), along with an excess of a vector expressing the bacterial effector SopE. Transfected cells were subsequently stained with rhodamine-phalloidin to visualize the actin cytoskeleton. Note the absence of membrane ruffles in cells transfected with SopE and Cdc42Hs^{C40} (green cell, left panel) and abundant ruffles in cells transfected with SopE and the empty vector expressing GFP (arrows on green cell, right panel).

specialized protein secretion and translocation apparatus termed type III, encoded at centisome 63 of the *S. typhimurium* chromosome (3). Small GTPases of the Rho subfamily have been implicated in a wide variety of cellular functions, including the organization of the actin cytoskeleton, the assembly of focal adhesion complexes, cytokinesis, and cell growth and differentiation (42). The actual mechanisms by which this family of small G proteins modulates such a large variety of cellular functions are poorly understood, although it is assumed that they exert their various functions by engaging different downstream effectors. Several putative effectors of Cdc42 and Rac have been identified using a variety of biochemical or genetic approaches. In most instances, these putative effectors have been identified by exploiting their ability to bind these Rho GTPases in a GTP-dependent manner. The identified putative effectors are either protein kinases such as PAK, activated Cdc42-associated kinase (ACK), and mixed lineage kinase 3 (MLK3), or, as in the case of IQGAP and WASP, proteins that contain domains suggestive of their involvement in signal transduction by protein–protein interactions (16). The identification of putative effector proteins has been complemented by the definition of specific domains or effector loops in the GTPases themselves that are thought to specifically mediate their functional linkage to specific downstream signaling pathways or cellular responses.

In this report, we have investigated the potential role of PAK, a putative effector of Cdc42, in *S. typhimurium*–induced cellular responses. Infection of cultured cells with wild-type *S. typhimurium* resulted in a significant stimulation of PAK activity. The stimulation was rapid and short-lived, with peak kinase activity 10 min after infection and a rapid decline shortly thereafter. PAK activation was strictly dependent on the delivery of effector proteins through the type III protein secretion system, since a *S. typhimurium invG* mutant, which is deficient for this system, failed to activate PAK activity. Expression of a dominant-negative kinase-deficient PAK mutant blocked JNK activation, indicating

that the kinase activity of PAK is required for *S. typhimurium*–induced nuclear responses. The inhibitory effect of the kinase-defective mutant is unlikely to have been due to a nonspecific sequestration of Cdc42, since the same construct did not block other Cdc42-dependent responses, such as bacterial internalization. The specificity of this effect is further demonstrated by the finding that expression of a kinase-defective mutant of MLK3, another effector target of Cdc42, did not significantly block *S. typhimurium*–induced JNK activation (Chen, L.-M., and J.E. Galán, unpublished results). The *S. typhimurium*–induced JNK activation as a consequence of the stimulation of PAK is consistent with previous reports that have shown that expression of constitutively active PAK resulted in JNK activation (37, 43). In contrast to the nuclear responses, the actin cytoskeleton rearrangements induced by *S. typhimurium* were not dependent on the kinase activity of PAK. Expression of a kinase-defective PAK did not result in inhibition of actin cytoskeleton–mediated *S. typhimurium* internalization into host cells. These results clearly demonstrate that the *S. typhimurium*–induced cellular responses are dependent on different downstream Cdc42 effector activities. However, previous reports have shown that PAK modulates the organization of the actin cytoskeleton via kinase-independent mechanisms (28). Those reports have implicated certain proline-rich domains at the NH_2 terminus of PAK that are postulated to be involved in the binding of SH3 domains in downstream effector proteins. In particular, studies have identified a proline-rich motif between amino acids 11 and 16 of PAK that is essential for the modulation of actin cytoskeletal organization. This motif has also been implicated in the binding of the SH3-containing adapter protein Nck (41). Therefore, we investigated the potential role of these NH_2-terminal proline-rich domains of PAK in *S. typhimurium* internalization into host cells. Expression of PAK mutants carrying specific mutations in each of these proline-rich regions did not impair actin cytoskeleton–mediated bacterial internalization. These results suggest that PAK may not be required for actin cytoskeleton responses stimulated by *S. typhimurium*. However, it is possible that expression of such mutants may not result in an adequate dominant-negative effect, or PAK may contain other domains that may be involved in bacteria-induced cytoskeletal responses.

Transient expression of an effector loop mutant of Cdc42 ($Cdc42^{C40}$) unable to bind CRIB domain–containing proteins resulted in effective inhibition of both *S. typhimurium*–induced nuclear and actin cytoskeleton responses. These results indicate that both responses required effectors that interact with this domain of Cdc42. PAK contains a CRIB domain and is therefore impaired in binding to this effector loop mutant of Cdc42. Thus, the dominant-negative effect of $Cdc42Hs^{C40}$ on the nuclear responses induced by *S. typhimurium* is consistent with our findings that PAK activity is required for bacteria-induced JNK activation. However, it is unclear whether a potential requirement for PAK may explain the effect of $Cdc42Hs^{C40}$ on *S. typhimurium*–induced cytoskeletal rearrangements, as we failed to demonstrate the involvement of this kinase on the bacteria-induced morphological response. Further studies will be required to address this question and to identify other effectors of Cdc42 that may be required for nuclear and cytoskeletal responses.

Our results showing the requirement of the CRIB-binding domain of Cdc42 for the actin cytoskeleton reorganization induced by *S. typhimurium* are not in full agreement with previous studies with effector loop mutations of Cdc42 that have argued that CRIB-containing effector proteins do not mediate actin cytoskeleton responses modulated by this small G protein (32). However, this discrepancy may be due to the different experimental set-ups. In our studies, the activation of Cdc42 to induce cellular responses is mediated by a bacterial effector that directly stimulates this small G protein. In contrast, other studies have made use of a constitutively active Cdc42 mutant carrying the effector loop substitutions (e.g., $Cdc42Hs^{L61C40}$; reference 32). Most likely, the constitutive activation of this GTPase is not equivalent to the *S. typhimurium*–mediated stimulation of Cdc42, which is transient. Thus, activation of this GTPase mediated by the bacterial agonist may lead to interactions with downstream effectors that are different from those resulting from its constitutive, irreversible activation by introduction of an activating mutation.

The results described here show that PAK is activated upon *S. typhimurium* infection of host cells. This activity is required for bacteria-induced nuclear responses, as expression of a kinase-defective PAK^{R297} mutant blocked both *S. typhimurium*– and SopE-mediated JNK activation. In contrast, this mutant did not block actin cytoskeleton–mediated *S. typhimurium* entry into host cells, indicating that the nuclear and morphological responses stimulated by the bacteria are mediated by different Cdc42Hs downstream effector activities. Expression of $Cdc42Hs^{C40}$, which is defective for binding to PAK and other effectors containing a CRIB domain, blocked both *S. typhimurium* nuclear and cytoskeletal responses, implicating this effector loop of Cdc42 in mediating both responses.

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