EhPAK, a member of the p21-activated kinase family, is involved in the control of *Entamoeba histolytica* migration and phagocytosis

Elisabeth Labruyère[1,*], Christophe Zimmer[2], Vincent Galy[1,‡], Jean-Christophe Olivo-Marin[2] and Nancy Guillén[1]

[1]Unité de Biologie Cellulaire du Parasitisme, INSERM U389, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France
[2]Unité d'Analyse d'Images Quantitative, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France
*Author for correspondence (e-mail: elabruye@pasteur.fr)
‡Present address: Gene expression program, EMBL Heidelberg, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

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Summary

Entamoeba histolytica **migration is essential for the development of amoebiasis, a human disease characterised by invasion and destruction of tissues. Amoebic motility requires both polarisation of the cell and formation of a predominant pseudopod. As p21-activated kinases PAKs are known to regulate eukaryotic cell motility and morphology, we investigated the role of PAK in *E. histolytica*. We showed that the C-terminal domain of EhPAK comprised a constitutive kinase activity in vitro and that overproduction of this fragment, in *E. histolytica*, caused a significant reduction in amoeboid migration, as measured by dynamic image analysis, indicating an involvement of EhPAK in this process. A dramatic loss of polarity, as indicated by the increased number of membrane extensions all around *E. histolytica*, was also observed, suggesting that the N-terminal domain of EhPAK was necessary for maintenance of cell polarity. To support this view, we showed that despite the absence of the consensus motif to bind to Rac and Cdc42, the N-terminal domain of EhPAK bound to Rac1, suggesting that the N-terminal region was a regulatory domain. In addition, we also found an increased rate of human red blood cell phagocytosis, suggesting for the first time an active role for a PAK protein in this process. Taking together, the results suggest strongly that EhPAK is a key regulatory element in polarity, motility and phagocytosis of *E. histolytica*.**

Key words: Amoebiasis, Pseudopod, Serine/threonine kinase, Quantitative imaging, Rac

Introduction

Many eukaryotic cell types require regulated motility in order to achieve specific biological events including embryonic development, immune response, wound healing and tumour cell metastasis. Cell motility is determined by three key well coordinated features: (i) polarisation of cell morphology, (ii) adherence to and detachment from a substratum and (iii) the capacity to generate forces for translocation. The dynamic reorganisation of the actin cytoskeleton plays a crucial role in all these processes (for a review, see Mitchison and Cramer, 1996). The Rho family of small GTPases, such as Rho, Cdc42 and Rac, are well known regulators of such changes (Hall, 1998). The p21-activated serine/threonine protein kinases (PAKs) are effectors for the small GTPases Rac and Cdc42 but not for Rho (Nobes and Hall, 1999). These kinases mediate specific effects of Rac and Cdc42 on actin organisation, adhesion, gene transcription and cell motility (for a review, see Bagrodia and Cerione, 1999) (Kiosses et al., 1999; Sells et al., 1999). All members of the PAK family have a highly conserved motif, located at the N-terminal region, called the p21-binding domain (PBD) or the Cdc42/Rac interactive binding (CRIB) domain (Burbelo et al., 1995). The N-terminal domain also displays proline-rich sequences that potentially binds to Src homology 3 (SH3) domains such as the adaptor Nck (Galisteo et al., 1996) and the guanine nucleotide exchange factor PIX (Pak-interacting exchange protein) (Manser et al., 1998; Bagrodia et al., 1998). The C-terminal catalytic domain of PAK is well conserved across species. Homologues of mammalian PAKs have been described in unicellular organisms. In *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, PAK enzymes are involved in morphogenic processes that control cell polarity and actin cytoskeleton organisation (Barylko et al., 2000; Eby et al., 1998). In the amoebae *Acanthamoeba castellanii* and *Dictyostelium discoideum*, members of the PAK family, named myosin I heavy chain kinases (MIHCK), are well described (Brzeska et al., 1996; Lee et al., 1996). More recently, a putative PAK, PAKa, has been described in *D. discoideum* and plays a role in directional movement, suppression of lateral pseudopodia extension and proper retraction of the posterior of cells undergoing chemotaxis (Chung and Firtel, 1999).

A highly motile amoeba, the pathogenic protozoan parasite *Entamoeba histolytica*, is the causative agent of human amoebiasis, a wide spread infectious disease whereby the amoeba invade and destroy human intestine and liver. The motility of this parasite is critical for its pathogenicity, which is reflected by tissue invasion, dissemination and development of acute disease. Motile *E. histolytica* trophozoites are

polarised with an anterior extended pseudopodia and a posterior appendage called uroid, which is composed of accumulated membrane (for a review, see Guillén, 1996). In *E. histolytica*, signal transduction mechanisms enhanced by external stimulation result in reorganisation of the actin cytoskeleton (Meza, 2000). Erythrophagocytosis, a pathogenic marker of *E. histolytica*, requires pseudopodia extensions and is dependent on the actin-myosin cytoskeleton dynamic (Voigt and Guillén, 1999). Proteins related to actin cytoskeleton dynamics such as tyrosine or serine/threonine kinases or small GTPases from the Rac and Rho subfamilies (Guillén et al., 1998; Lohia and Samuelson, 1993; Que et al., 1993) have been identified in *E. histolytica*. A Rac protein, Rac G, has been shown to be involved in the signal transduction pathway controlling capping of surface receptors and uroid formation, as well as in distribution of F-actin, parasite polarity and cytokinesis (Guillén et al., 1998). A gene encoding a polypeptide, named EhPAK, that shares homology with the murine p21-activated kinase and the yeast Ste20 was also described (Gangopadhyay et al., 1997). Given the lack of knowledge of transduction pathways leading to cell polarization, motility and phagocytosis in *E. hitolytica*, we decided to investigate EhPAK with regard to its potential role in these processes by biochemical and cell biology approaches. The C-terminal domain of EhPAK, carrying a constitutively kinase activity, was overproduced in *E. histolytica* and caused a loss of polarity, a decrease in motility and an enhancement of red blood cell phagocytosis. Although EhPAK does not contain a CRIB motif, the N-terminal domain of EhPAK was able to bind to the small GTPase Rac1. Taken together, our data suggest that N-terminal part of EhPAK is a regulatory domain and that EhPAK is a major player in the signalling pathway that controls *E. histolytica* polarity, motility and phagocytosis of human cells.

Materials and Methods

Strains and culture conditions

Pathogenic *E. histolytica* strain HM1: IMSS was cultivated axenically in a TYI-S-33 medium (Diamond et al., 1978) at 36°C. The MyoIB$^+$ strain (Voigt et al., 1999) was cultured in the same medium in the presence of G418 (10-50 μg/ml). *Escherichia coli* DH5α and BL21 (DE3), used for plasmid construction and for recombinant PAK production respectively, were grown in Luria-Bertani medium (Sambrook et al., 1989) in the presence of ampicillin at 100 μg/ml when required.

Cloning of the *pak* gene, truncated *pak* gene amplification and purification of recombinant proteins

The ORF corresponding to the *pak* gene was amplified from *E. histolytica* genomic DNA by PCR using two oligonucleotide primers based on the published *pak* gene sequence (Gangopadhyay et al., 1997). (Sense primer: 5′*GGGAATTCCAT***ATGGTTTCATGTAAAAAACATGG**3′, which created a *Nde*I site and an antisense primer that created a *Xho*I site: 5′CCGC*CTCGAG***AATACCTACTTTCATTCCTCTTTG**3′). From five distinct PCR isolates a 1.4 kb fragment was obtained, cloned into pUC19 and sequenced. The deduced sequence of *E. histolytica* PAK amino acids was compared with protein sequences in the National Center for Biotechnology Information. To obtain GST hybrid proteins, the entire and truncated *pak* genes were amplified by PCR, subcloned in the prokaryotic gene fusion vector pPGEX-4T-2 and expressed in *E. coli* as follows. The 5′ primers created an *Eco*RI site, the 3′ primers created a *Xho*I site. To amplify the *pak* gene, the oligonucleotide sense, OS1, 5′GATC*GAATTC***ATGGTTTCATGTAAAAAACATGG**3′ and the antisense, OR 1374, 5′GATC-*CTCGAG***TTAAATACCTACTTTCATTCCTC**3′ primers were used. To amplify both the DNA encoding N-PAK (amino acids 1-150) and the DNA encoding Np-PAK (amino acids 1-186) the 3′ primer used was OS1 and the 5′ antisense primers were 5′ GATC*CTCGAG***TCTTTTCTCTGTAACCGTAG** 3′ for N-PAK and 5′ GATC*CTCGAG***TGGGTCTTCTGTTTTACTATATC** 3′ for Np-PAK. A DNA fragment encoding amino acids 187-457 (C-PAK) was amplified with 5′ primer (sense: 5′GATC*GAATTC***CATAAATATTTTACAAATCTTGTTTC**3′ and 3′ primer antisense OR1374). The amplified products were digested and then cloned into the pPGEX-4T-2 cleaved with *Eco*RI and *Xho*I. Hybrid proteins GST-PAK, GST-N-PAK, GST-Np-PAK and GST-C-PAK, were expressed in *E. coli* BL21 cells and purified on glutathione-coupled Sepharose beads as described in the instructions (Amersham Pharmacia Biotech).

In vitro kinase assay

Sepharose glutathione beads alone or loaded with GST-C-PAK (1 μg) and the resulting soluble C-PAK (500 ng) after a thrombin cleavage were incubated in 40 μl of kinase buffer (40 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 4 mM $MnCl_2$) containing 10 μg of myelin basic protein (0.25 μg/ml), 10 μM ATP and 2 μCi of [γ-^{32}P] ATP for 20 minutes at 30°C. Kinase assays were stopped by addition of sample buffer, boiled for 5 minutes, and proteins separated by a 15% SDS-PAGE for transfer to nitrocellulose were analysed by phosphorimaging.

Binding to Rac or to CDC42 assay

GST-truncated PAK proteins were bound to glutathione beads and incubated with purified Rac1 or Cdc42. *E. coli* producing GST-Rac1 or GST-Cdc42 were kindly provided by A. Hall, (University College London, London, UK) and *E. coli* producing GST N-WASP were a gift from C. Egile (our laboratory). The small GTPases and N-WASP were purified on glutathione-coupled Sepharose beads. To obtain active GTPases, GST-Rac1 and GST-Cdc42 bound to glutathione beads were depleted from nucleotide by incubation in 50 mM Tris HCl pH 7.6, 100 mM NaCl, 1 mM DTT and 2.5 mM EDTA for 1 hour at room temperature. GST-Rac and GST-Cdc42 were then loaded with GDP or GTPγS in 50 mM Tris HCl pH 7.6, 100 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$ and 1 mM GDP or GTPγS for another hour and then cleaved with thrombin. The resulting proteins were analysed by western blot using monoclonal anti GST (kindly provided by C. Egile) and either an anti Rac or an anti Cdc42 antibody to confirm that the GST-hybrid was not present. 1.5 μg of Cdc42 or Rac1 (GDP or GTPγS loaded) were incubated with the GST-fusion proteins GST-PAK (0.5 μg), GST-N-PAK (5 μg), GST-Np-PAK (5 μg) and GST-C-PAK (5 μg) bound to glutathione-coupled Sepharose beads. After three washes, beads were suspended in SDS-PAGE sample buffer, boiled for 5 minutes, then proteins separated on a 12% acrylamide gel and transferred to a nitrocellulose membrane. The interaction between GST-hybrids proteins and GTPases were examined by anti-GST, anti-Rac1 and anti-Cdc42 immunoblots.

Immunoblotting and antibodies

Samples were boiled for 2 minutes in SDS-sample buffer containing 2% 2β mercaptoethanol, electrophoresed on 12% SDS-PAGE gels and transferred to PVDF or to nitrocellulose membranes by semi-dry electrophoretic transfer in Tris-glycine buffer. Western blots were performed as described previously (Arhets et al., 1998). Specific proteins were detected using the appropriate antibodies: monoclonal anti human Rac1 (Upstate Biotechnology) (diluted 1:1000); monoclonal anti human Cdc 42 (Santa Cruz Biotechnology, Inc)

(diluted 1:2000); monoclonal anti actin (mAb N350; Amersham Pharmacia Biotech) (diluted 1:2000). To produce polyclonal serum to PAK (this work) rabbits were immunised with recombinant C-PAK and with entire PAK. Antibodies were affinity purified by elution from purified proteins transferred to a nitrocellulose support. Secondary antibodies, peroxidase-labelled anti-rabbit IgG or anti-mouse IgG (Nordic Immunology) (1:20000) were used, and immunoreaction was detected with ECL or ECL plus kits detection methods (Amersham Pharmacia Biotech). Immunoblots were exposed to X-OMAT film (Eastman Kodak Company) or to a phosphor screen for general purposes (Eastman Kodak Company) when necessary to quantify the bands with the IQ Mac V12 molecular imaging system.

Immunofluorescence and confocal microscopy

Trophozoites incubated at 36°C in amoebae culture medium without serum on coverslips were fixed with 3.7% paraformaldehyde (PFA), permeabilised with 0.1% Triton X100/PBS, blocked with 1% BSA/PBS for 30 minutes, washed and incubated with purified IgG anti-C-PAK (1:20 dilution) in 1% BSA/PBS for 1 hour. Coverslips were washed and incubated for 30 minutes with CY3-labelled rabbit antibodies (diluted 1:300) in 1% BSA/PBS (Jackson Immuno Research Laboratories, Inc.). F-actin was stained with FITC Bodipy phallacidine (Sigma Chemical Co.) Samples were analysed with an Axiovert 100M- Zeiss piloted by a LSM 510 confocal laser scanning microscope. Observations were performed in 15 to 20 planes from the bottom to the top with a thickness of 1 μm per optical section.

DNA plasmid transfection into *E. histolytica*

For overexpression experiments in *E. histolytica*, the gene encoding PAK cloned in pUC19 was amplified by PCR using a 5′ primer (sense: 5′ GATC*GGTACC***ATGGTTTCATGTAAAAAACATGG** 3′) that created a *Kpn*1 site and 3′ primer (antisense: GATC*GGATCC*-**TTAAATACCTACTTTCATTCCTC** 3′) that created a *Bam*HI site, and the fragment encoding C-PAK (the last 273 amino acids), cloned into pUC19, was amplified with a 5′ primer beginning at oligonucleotide 559 and creating a *Kpn*I site (sense: 5′ GATC*GG-TACC*ATGCATAAATATTTTACAAATCTTGTTTCTATTG 3′) and a 3′ primer creating a *Bam*HI site (antisense: 5′ GATC*GGATCC*-TTAAATACCTACTTTCATTCCTC 3′). The PCR products were digested by *Bam*HI and *Kpn*I and then cloned into the pExEhNeo plasmid, derived from pEhNEo/CAT (Hamann et al., 1995) and kindly provided by E. Tannich (Bernhard Nocht Institute for Tropical Medicine, Hambourg, Germany) at the *Bam*HI and *Kpn*I sites. pExEhNeo contains a gene conferring G418 resistance as a selectable marker and in this plasmid. The ORF-encoding PAK and C-PAK are flanked by 485 bp of untranslated 5′ sequence of an *E. histolytica* lectin gene and 600 bp of the untranslated 3′ sequence of an *E. histolytica* actin gene. Purification of the plasmids, transfection and selection of *E. histolytica* have already been described (Arhets et al., 1998; Guillén et al., 1998). In summary, the vector pExEhNeo and the recombinant plasmid (pExEhNeo/C-PAK) were replicated in *E. coli*, purified and transfected by electroporation in *E. histolytica* then grown in TYI-S-33 medium. After 48 hours, the transfected parasites were selected by their resistance to a medium supplemented with G418 (10 μg/ml). Amoeba expressing G418 resistance were cultured with G418 (3 μg /ml) and when necessary submitted to increasing concentrations of G418 up to 30 μg/ml.

Counting of *E. histolytica* protrusions

Membrane extensions of *E. histolytica* were analysed by confocal microscopy. Images obtained with differential interference contrast were superposed on images obtained by confocal laser-scanning fluorescence revealing F-actin staining at the posterior part of the parasite. Membrane protrusions were counted on roughly 50 amoeba of the control and C-PAK$^+$ strains.

Cell fractionation

After two days of culture, 10^6 trophozoites were allowed to adhere to the plastic bottom of a culture dish for 10 minutes in 2 ml of culture medium without serum and then were scrapped and centrifuged at 1200 ***g*** for 4 minutes at 37°C. The pellets were resuspended in 200 μl of cool PBS buffer containing an inhibitor cocktail EDTA free (Roche molecular Biochemicals), protease inhibitors (10 mM N-ehtyl-maleimide, 2 mM phenylmethylsulfonyl fluoride, 0.01 mM leupeptin and 2 mM parahydroxymercuribenzoate) and 1% Triton X100 and incubated for 30 minutes at 4°C. Debris and intact parasites were removed by low-speed centrifugation (1200 ***g***) at 4°C. The supernatants were submitted to a high-speed centrifugation (245,000 ***g***) for 30 minutes at 4°C. Pellets were washed in PBS and suspended in 200 μl of PBS buffer containing 1% SDS. The protein concentration of the supernatant was measured using the Bradford method, and for each sample an equal volume of the supernatant and the pellet fractions were analysed for their PAK and actin content by immunoblotting, and the bands were quantified with the IQ Mac V12 molecular imaging system.

Recording of *E. histolytica* migration and computer-assisted analysis of cellular movement

Prior to computer-assisted analysis, 5×10^5 amoeba, cultured in 30 μg/ml of G418, were transferred into a plastic flask full of culture medium without serum at 37°C. Adherent *E. histolytica* were filmed for 5 minutes in phase contrast mode with a video-camera mounted on top of an inverted-light-microscope. For each of the two strains (control, and C-PAK$^+$) a total of roughly 40 amoeba was analysed in three experiments. The video observations were digitised using a real-time digitisation system and decimated down to 1 frame per second, yielding sequences of 300 images for each experiment.

These image sequences were then processed for quantitative analysis with the help of a dedicated computer program based on the active contour approach (Kass et al., 1988), a widely used framework for image segmentation (identification of object regions) and tracking (following object motion in time series). In this method, the boundaries of each object (i.e. cells) are represented by a parametric curve $C(s,t)$, where s specifies position along the curve and t is a virtual time. Starting from an initial position $C(s,t=0)=C_0$, the curve is deformed according to an evolution equation:

$$\frac{\partial \mathbf{C}}{\partial t} = \alpha \frac{\partial^2 \mathbf{C}}{\partial s^2} + \nabla f,$$

where α is a positive coefficient and f is a so-called 'edge map', that is, an intermediate image computed from the original image in such a way that bright pixels strongly correlate with the locations of object boundaries. For phase contrast images, where cell boundaries have lower intensities than the background, a suitable edge map can be obtained by blurring the negative of the original image intensity field I with a Gaussian spread function G_σ of standard deviation σ: $f=-G_\sigma * I$ (* denotes convolution). The term ∇f in the equation above tends to push the contour towards object edges, whereas the other term acts to enforce contour smoothness and helps in overcoming image noise. The evolution process proceeds until convergence and the final curve was used to define the detected object boundaries. Thanks to several improvements (Zimmer et al., 2001), we were able to overcome important shortcomings of the original framework (Kass et al., 1988), in particular in detecting low contrast boundary deformations such as pseudopodia and in segmenting objects in contact (Zimmer et al., 2001; Zimmer et al., 2002). Our program provided a description of individual cell contours in each image in the form of a list of

coordinates corresponding to the locations of contour points. The position of each cell was then computed from the mass centre of the region delimited by the detected cell contour. The cell trajectories were readily obtained from the consecutive positions of these centres.

Spectrophotometric haemoglobin assay

To quantify the number of red blood cells (RBCs) ingested by the amoeba, a colorimetric determination of internalised haemoglobin was used. The method was described by Voigt and collaborators (Voigt et al., 1999). Briefly, RBCs from a healthy human volunteer was washed three times (2 minutes, 300 ***g***) with amoeba culture medium TYIS-33 to eliminate the serum and resuspended at $1\text{-}10\times10^8$ cells/ml. *E. histolytica* were washed with the same culture medium (2 minutes, 1000 ***g***) and pelleted at a concentration of $1\text{-}10\times10^6$cells/ml. Amoeba (2.4×10^5) were incubated with RBCs ($2.4\times10^{7)}$ for 10 minutes at 37°C in 0.2 ml of TYIS-33. The amoeba and RBCs were then spun down (15 seconds, 8000 ***g***) and twice resuspended in 1 ml of cold distilled water in order to burst non-ingested RBCs then centrifuged (15 seconds, 8000 ***g***). The pellet containing amoeba with RBCs completely engulfed was resuspended in 1 ml concentrated formic acid. Samples were measured with a spectrophotometer at 400 nm.

Results

Cloning of EhPAK and comparison with members of the PAK family

The *pak* gene was cloned by PCR (see Materials and Methods), and five independent clones were sequenced. The five nucleotide sequences obtained were identical. Nevertheless, a few differences attributed to sequencing errors were found after comparison with the published *pak* gene sequence, which is present in one single copy in the *E. histolytica* genome (Gangopadhyay et al., 1997) (Fig. 1A). The validity of our DNA sequence was also confirmed by BLASTN comparison with genomic clones obtained during the genome project of *E. histolytica* (The Institute Genomic Research, TIGR). The isolated *pak* gene carried an open reading frame of 457 amino acids with a predicted molecular mass of 53 kDa. Analysis of the encoded amino-acid sequence by the Genetics Computer Group, Inc. program revealed motifs indicating that the protein was a potential PAK family member. For example, the C-terminal part of the protein contained serine/threonine kinase motifs (amino acids 307-317) and an ATP-binding site (amino acids 197-220), suggesting a putative catalytic domain (Fig. 1B). In addition, the putative regulatory N-terminal domain contained a polybasic region potentially required for Rac binding and enzyme activation (Knaus et al., 1998) and a potential SH3-interacting polyproline stretch followed by an acidic stretch (Fig. 1B). Surprisingly, EhPAK did not possess the consensus CRIB motif (Fig. 1B). The deduced amino-acid sequence of EhPAK was compared with available sequences in the National Center for Biotechnology (NCBI) by a BLAST search. The EhPAK kinase domain showed identity to the kinase domain of human PAK2 (36% identity, 58% similarity) (Fig. 1B), human PAK1 (35% identity, 45% similarity), *Dictyostelium discoideum* MICH (28% identity, 50% similarity) and *D. discoideum* PAKa (60% identity, 71% similarity). In addition, the C-terminal domain presented 3D structural homology with the catalytic domain of serine/threonine kinase PAK1 (accession number 1F3M, CD-search by BLASTP at NCBI pdb library).

Binding of human small GTPases Rac1 and Cdc42 to *E. histolytica* PAK

To analyse whether EhPAK was a member of the p21-activated kinase family despite the absence of the CRIB domain, binding assays were performed with the GTPases Rac and/or Cdc42 (Fig. 2A). We decided to conduct these experiments with human GTPases, since no Cdc42 has been described in *E. histolytica* and human Rac1 present 99% homology to RacG from *E. histolytica* (Guillén et al., 1998). As the full-length EhPAK produced in *E. coli* was unstable and easily degraded, only truncated forms N-PAK (amino acids 1 to 150), NpPAK (amino acids 1 to 186) and C-PAK (187 to 457) were purified as GST hybrid proteins and used in binding assays. These proteins were incubated in the presence of human Rac1 and Cdc42 in an inactive form loaded with GDP or in an active form loaded with the non-hydrolyzable GTP analogue GTPγS. GTPases were incubated with beads coated with different PAKs and control peptides (Fig. 2A). Since it is well known that the Wiskott-Aldrich-Syndrome Protein (WASP) binds Rac and Cdc42 in vitro, the truncated WASP protein containing the CRIB motif was included as a positive control and the purified GST protein as a negative control. The proteins binding the beads were then electrophoresed and blotted. The capacity of the p21 GTPases to bind to the different PAK constructs was determined by western blot analyses with antibodies against Rac1 and antibodies against Cdc42 (Fig. 2A). The amount of different PAK GST-hybrid proteins was verified by monoclonal antibodies against GST (data not shown). The major result obtained in these experiments is that a Rac-binding site is located in the N-terminal domain of EhPAK, suggesting that this region could be the regulatory domain of the enzyme. EhPAK seemed to bind exclusively the active form of Rac1 (GTP/Rac1) and did not bind GDP/Rac1 or Cdc42 (GTP/Cdc42 or GDP/Cdc42). These results contrasted with those obtained from the analysis of other PAKs, which interacted via a CRIB motif with both activated Rac and Cdc42 (Burbelo et al., 1995).

Kinase activity of PAK from *E. histolytica*

We were not yet able to preserve the stability of the full-length enzyme *in E. coli* to reveal the kinase activity of the entire EhPAK, as well as its putative regulation by a Rac protein. To determine whether EhPAK had a kinase activity, we chose to carry out the myelin basic protein (MBP) kinase assay on the protein with its N-terminal domain deleted. Samples were run on a gel, analysed with a phosphoimager and the phosphorylation was quantified in arbitrary units with the IQ Mac V12 molecular imaging system. Both recombinant GST-C-PAK bound to beads and soluble C-PAK (amino acids 187-457), containing the putative kinase catalytic domain, were utilised (Fig. 2B). In comparison with the residual background (Fig. 2B, lane 3) there was a major increase in MBP phosphorylation with both beads-GST-C-PAK (a twofold increase) and C-PAK (a fourfold increase) (Fig. 2B lane1 and lane 2, respectively), demonstrating that EhPAK possessed a kinase activity and, in addition, that N-terminal deletion yielded a constitutively active fragment able to phosphorylate MBP as a substrate.

Cellular distribution of PAK in *E. histolytica*

Since PAKs are known to regulate actin organisation in

mammalian cells and in amoeba such as *D. discoideum*, the distribution of both EhPAK and the filamentous actin were examined together by laser confocal microscopy in rounded parasite and in elongated migrating *E. histolytica*. PAK was revealed using a polyclonal antibody affinity purified using the last 271 amino acids of the protein (Materials and Methods). This serum recognised a unique *E. histolytica* peptide at 53 kDa (Fig. 5B). Polymerised actin was co-stained with fluorescent phallacidin. In rounded amoeba, PAK and F-actin were dispersed throughout the cytoplasm (unpublished results). In moving trophozoites, PAK redistributed to the anterior part of the cell and became highly concentrated in the nascent pseudopod (Fig. 3). Polymerised actin, moderately abundant in the cytoplasm, was present in the forming pseudopod and at the periphery of the cell. In contrast to the localisation of PAK, F-actin was in addition distributed in increased abundance at the posterior pole of the amoeba (Fig. 3). Thus, in moving parasites, the concentrated labelling at opposite ends of PAK versus F-actin reinforced the polarised state of migrating *E. histolytica*.

A

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EhPAK(1) 155 QLQQKIEHPQAHIEKPKKEPPPEEDDLLRYSKTEDPHKYFTNLVSIGKGG 204
             ||||||  . . .  .|| ..|||||||||:||||:||||||||||||||||
EhPAK(2) 151 QLQQKIRTSTSSYCFSKKRTSPEEDDLLRYCKTEEPHKYFTNLVSIGKGG 200

EhPAK(1) 205 FGEVFC.AQRIEDGKPIALKMLKHSVDERYTKIGAEVARLSNWKHPNIVG 253
             |||||:.|||||||||:.||||||||||||||||||||||||||||||||
EhPAK(2) 201 FGEVFSLTQRIEDGKPIRFKMLKHSVDERYTKIGAEVARLSNWKHPNIVG 250
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B.

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EhPAK     1        MVSCKKHGKTFWARRSLNIYNGLIALYAVNGEIPLTILPFSD 42

HsPAK2    1                                  MSDNGELEDKPPAPPVR 17

EhPAK    43 NVEITKHQRLILIFQCVIRRVYLRFETEEECNEVETLCKKCQQYYHKPQT 92

HsPAK2   18 MSSTIFSTGGKDPLSANHSLKPLPSVPEEKKPRHKIISIFSGTEKGSKKK 67
                                                             ||:
EhPAK    93 MIVKDFFSGKLADLTPTCRELCNKNNLYPEECEKHFDTLCEILRSQTKKR 142

HsPAK2   68 EKERPEISPPSDFEHTIHVGFDAVTGEFTGMPEQWARLLQTSNITKLEQK 117

EhPAK   143 YATVTEKRQLQQKIEHPQAH.............................. 162

HsPAK2  118 KNPQAVLDVLKFYDSNTVKQKYLSFTPPEKDGLPSGTPALNAKGTEAPAV 167

EhPAK    ..................................................

HsPAK2  168 VTEEEDDDEETAPPVIAPRPDHTKSIYTRSVIDPVPAPVGDSHVDGAAKS 217

EhPAK   163 IEKPKKEPPPEEDDLLRYSKT....EDPHKYFTNLVSIGKGGFGEVFCAQ 208
            ::| ||.|   ::::: :|    || | :|    ||.|  ||| |
HsPAK2  218 LDKQKKKPKMTDEEIMEKLRTIVSIGDPKKKYTRYEKIGQGASGTVFTAT 267

EhPAK   209 RIEDGKPIALKMLKHSVDERYTKIGAEVARLSNWKHPNIVGFEGCWLYNN 258
             :   |.:|:| :      :   |  |: :   |.|||| |   :|  .
HsPAK2  268 DVALGQEVAIKQINLQKQPKKELIINEILVMKELKNPNIVNFLDSYLVGD 317

EhPAK   259 QVYIGMEYCSLGTLKTLLKNRMRPFQDADTAFLLLETLKALKYIHEEGFI 308
            :.:: |||  .|.| | .        :|  | .  | |.||.::|    |
HsPAK2  318 ELFVVMEYLAGGSL.TDVVTETACMDEAQIAAVCRECLQALEFLHANQVI 366

EhPAK   309 HRDIKTANIMLKQNFEIKVIDFGLVVRI...NSKPSNRAGSKAYMTPEVI 355
            |||||. |::|     :|. |||   .|    || |   |. :|  |||:
HsPAK2  367 HRDIKSDNVLLGMEGSVKLTDFGFCAQITPEQSKRSTMVGTPYWMAPEVV 416

EhPAK   356 KQIPYNEKVDIWSIGCVAQELFEGSPPYRELGMFKGLFKTATVGAPGLRN 405
             .  |  ||||||:|. | |: || |||     :  |: ||  | | |.|
HsPAK2  417 TRKAYGPKVDIWSLGIMAIEMVEGEPPYLNENPLRALYLIATNGTPELQN 466

EhPAK   406 PSKAPPELVDFLKKCFFFNPEERWSATQLLEHPFLKQADGSIIKQRGMKV 455
            | |  |   ||| :|   . |.| || :||:||||| |
HsPAK2  467 PEKLSPIFRDFLNRCLEMDVEKRGSAKELLQHPFLKLAKPLSSLTPLIMA 516

EhPAK   456 GI
HsPAK2  517 AKEAMKSNR
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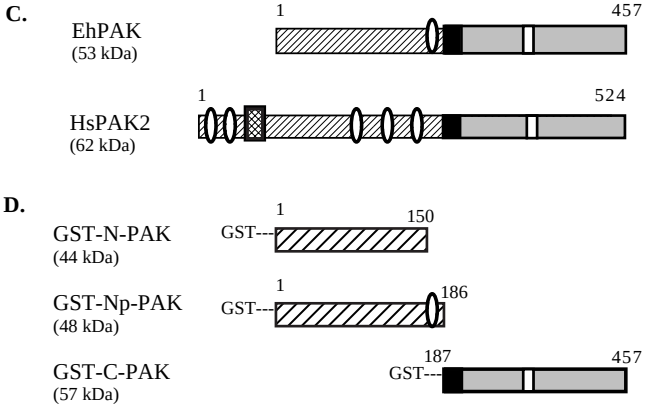

Fig. 1. Molecular description of EhPAK. (A) The EhPAK region containing the few differences observed between the EhPAK (1) sequence (this paper) and the EhPAK (2) sequence (Gangopadhyay et al., 1997). (B) Amino-acid sequence comparison of EhPAK to the human PAK2 (HsPAK2). The N-terminal sequence of EhPAK shows a polybasic sequence (bold and italic) and a proline-rich sequence (italic) and the C-terminal sequence contains a predicted ATP-binding site (bold and underlined) and catalytic site (bold). The regulatory N-terminal sequence of human PAK2 contains the Nck-binding motif (bold), the polybasic region (italic), the CRIB domain (italic, bold and underlined) and the PIX interacting sequence (grey). The catalytic domain of HsPAK2 showing the ATP binding site (bold and underlined) and the catalytic site (bold). Accession numbers: EhPAK (1) EMBL X98048 and HsPAK2 (u24153.gb_pr). Sequence comparisons were performed with BESTFIT function in the GCG program. (C) Schematic representation of EhPAK and HsPAK2 showing their N-terminal region (oblique lines) and their catalytic domain (grey). The proline-rich sequence (open circle), CRIB motif (hatched box), ATP-binding site (black box) and catalytic site (white box) are indicated. (D) Shematic representation of the GST-hybrid fusion proteins produced in *E. coli* and purified.

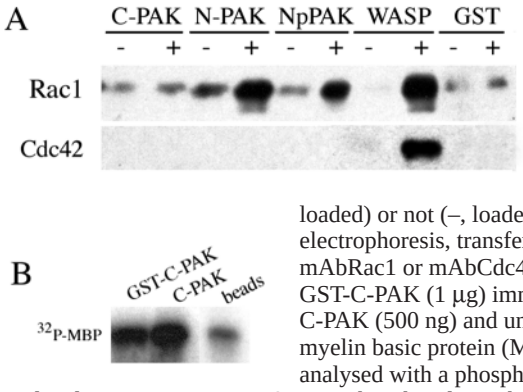

Fig. 2. Biochemical characteristics of EhPAK. (A) Interaction of truncated EhPAK with small GTPases Rac1 or Cdc42 GST-hybrid proteins carrying –C-PAK (5 μg), –N-PAK (5 μg), –Np-PAK (5 μg) and –WASP (0.5 μg) coupled to glutathione Sepharose beads were incubated with purified recombinant GTPases (1.5 μg), human Rac1 or human Cdc42, activated (+, GTPγS loaded) or not (–, loaded with GDP). The potential complex (hybrid protein)/GTPase was separated by electrophoresis, transferred to nitrocellulose, and the presence of GTPases was revealed by immunoblotting with mAbRac1 or mAbCdc42 antibodies. (B) Kinase activity of the C-terminal domain of EhPAK. The hybrid protein GST-C-PAK (1 μg) immobilised on glutathione Sepharose beads and thrombin-cleaved derivative soluble protein C-PAK (500 ng) and uncoated glutathione Sepharose beads were subjected to an in vitro kinase assay using myelin basic protein (MBP) as the substrate in the presence of [γ-^{32}P]ATP. Samples were run on a gel and analysed with a phosphoimager, and the phosphorylation was quantified in arbitrary units with the IQ Mac V12 molecular imaging system. An autophosphorylation background of MBP is shown in the control line and a large increase in MBP phosphorylated was obtained when the carboxylic domain of EhPAK was added.

Construction of an *E. histolytica* strain overproducing the catalytic domain of EhPAK

To examine the role of EhPAK in vivo, and as genetic exchanges are not yet available in *E. histolytica*, we decided to engineer strains overproducing full-length or truncated EhPAK. Recombinant plasmids and the control carrier vector (ExEhNeo) were electroporated into amoeba, and transfected parasites were selected in the presence of G418 at 10 μg/ml (Materials and Methods). The following strains: N-PAK$^+$, C-PAK$^+$ and PAK$^+$, carrying, respectively, the DNA fragment encoding the N-terminal domain (amino acids 1-150), the C-terminal part (amino acids 187-457) of EhPAK and the entire protein, were obtained. Analysis of the protein content of these different transfectants, grown at 30 μg/ml of G418, revealed that the N-terminal peptide was unstable since it was not detectable with specific antibodies. For this reason the N-PAK$^+$ strain was not analysed further. The PAK$^+$ strain produced twofold more of the enzyme (data not shown) than the total endogenous PAK, and the C-PAK$^+$ strain produced an equivalent amount of the C-PAK fragment (Fig. 5B).

Cell morphology analysis of *E. histolytica* strain overproducing C-PAK

In mammalian cells, the PAK enzyme is a major regulator of cell morphology. To examine the role of EhPAK on the polarisation of *E. histolytica* in wild-type and transfected strains, we counted the number of pseudopodia on fixed amoeba (Materials and Methods). No noticeable morphology differences were observed between the control plasmid carrying strain (Fig. 4A,B), the PAK$^+$ strain (data not shown) and the wild-type *E. histolytica*. Roughly 90% of these trophozoites produced one to three pseudopodia, but only one of them became dominant, indicating the direction of locomotion (Fig. 4A,B). By contrast, we observed that cells from the C-PAK$^+$ strain displayed a dramatic change in morphology (Fig. 4A,B). In fact, 70% of the cells producing C-PAK exhibited four to six lateral extensions distributed all around the cell (Fig. 4A,B). Simultaneous protrusions of multiple pseudopod-like structures inhibited cell polarisation in the strain overproducing the constitutively active C-PAK. Thus, EhPAK appeared as a regulatory element controlling pseudopod extension and cell polarity.

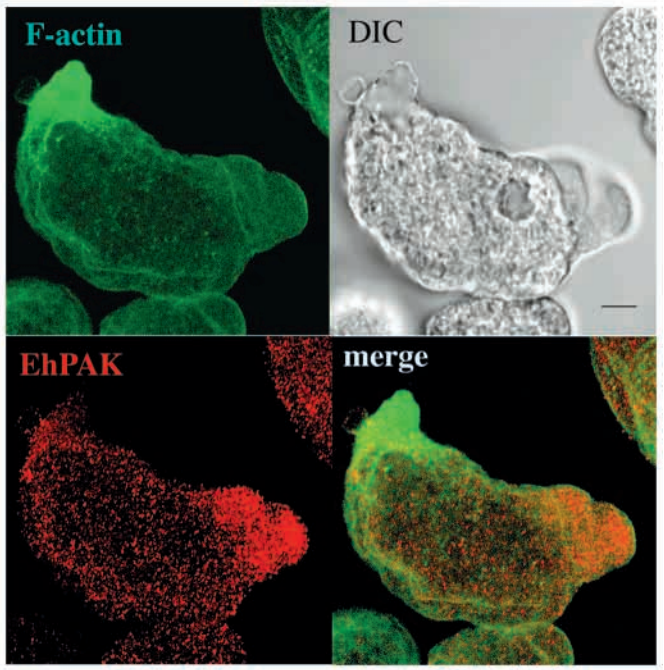

Fig. 3. Cellular distribution of EhPAK and F-actin in a motile *E. histolytica.* Amoeba moving from left to right display a prominent pseudopod that guides its locomotion. After fixation and permeabilisation, filamentous actin was decorated with FITC-phallacidine (green), and EhPAK was stained with purified polyclonal antibodies against EhPAK (red). A confocal micrograph of an optical plane in the middle of the parasite showed that F-actin was moderately concentrated in the pseudopod, distributed in the cytoplasm and enriched at the posterior end of the parasite. EhPAK was dispersed throughout the cytoplasm and was concentrated in pseudopodia extensions. Superimposition of the two stainings indicates that F-actin and EhPAK were concentrated at specific opposite sites of the parasite. The differential interference contrast (DIC) image shows *E. histolytica* with a pseudopod at the front of migration. Bar, 5 μm.

EhPAK and F-actin distribution in transfectant strains

E. histolytica motility requires a dynamic redistribution of the actin cytoskeleton to produce a polarised cell. To examine

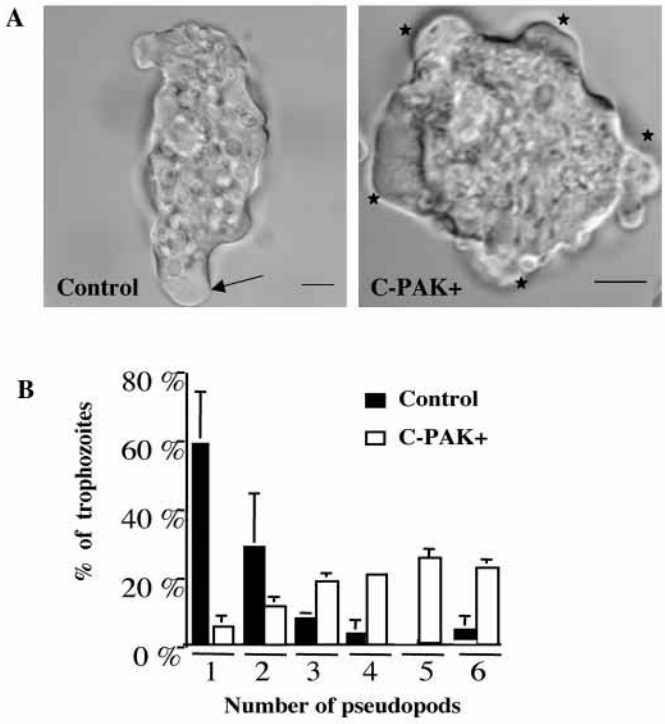

Fig. 4. Effect of EhPAK on *E.histolytica* morphology. The histograms represent the number of pseudopod counted for each strain. Fixed amoebae were examined by microscopy, and pseudopodia (indicated by the arrow) and/or pseudopodia-like structures (indicated by the stars) were counted as described in Materials and Methods. Note that the majority of control cells (90%) showed one to three pseudopodia. By contrast, 90% of the C-PAK$^+$ strain simultaneously displayed three to six protrusions.

whether overproduction of C-PAK had an impact on this process, the actin cytoskeleton was stained with fluorescent phallacidine. EhPAK and C-PAK were revealed by immunostaining with the polyclonal serum against EhPAK (Fig. 5A). Analysis by confocal microscopy showed that the distribution of EhPAK and F-actin in the control strain, containing the empty plasmid (Fig. 5A) and in the PAK$^+$ strain (data not shown) was identical to that already described for the wildtype. F-actin and EhPAK were highly concentrated at the opposite extremities of the migrating parasite, confirming that these cells displayed a wild-type phenotype. The C-PAK$^+$ strain that formed several pseudopod-like protrusions showed an enrichment of the PAK enzyme in these structures. In addition, we observed that there was no reinforced concentration of F-actin in this amoeba. This was in agreement with the lack of polarity of the C-PAK$^+$ strain.

Subcellular localisation of EhPAK and filamentous actin

We decided to analyse the potential link between EhPAK and actin microfilaments using a biochemical approach. Cellular fractionation was performed on both the control and C-PAK$^+$ strains to determine the subcellular localisation of F-actin and EhPAK. (Fig. 5B). The proteins present in the TritonX100-soluble fraction and in the TritonX100-insoluble cytoskeleton

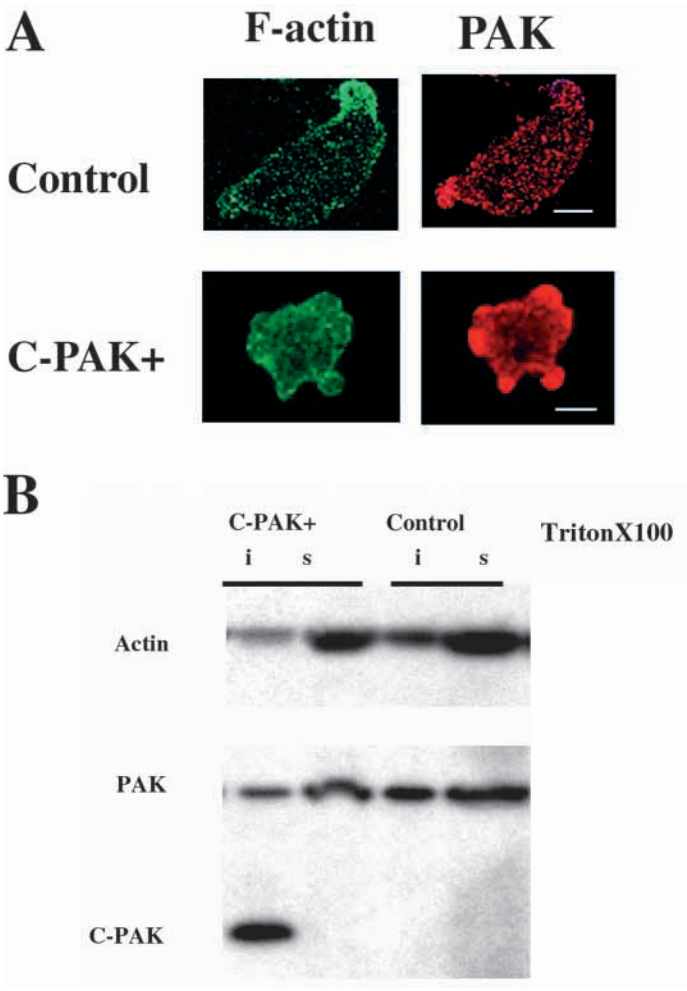

Fig. 5. Effect of EhPAK on F-actin distribution. (A) Micrographs represent confocal analysis of the different labelling obtained in optical planes through the middle of the cells. Moving amoebae (control and C-PAK$^+$) were fixed on cover slips, filamentous actin was decorated with phallacidine (green) and PAK was stained with specific anti-EhPAK polyclonal antibodies (red). As observed by DIC, the control strain was elongated, polarised and presented a unique pseudopod, and the strain overexpressing C-PAK is a rounded cell presenting multiple membrane extension. F-actin was diffusely distributed in the cytoplasm of the C-PAK$^+$ strain. For the control strain, F-actin was enriched at one pole of polarised parasites. EhPAK concentrated in the membranous protrusion for both C-PAK$^+$ and the control amoeba. Bars, 10 μm. (B**)** Cell fractionation in the presence of Triton X100 was performed on C-PAK$^+$ and control strains. The fractions were electrophoresed and analysed by western blot with anti-PAK or anti-actin antibodies. The revealed protein was analysed with a phosphoimager, bands were quantified in arbitrary units with the IQ Mac V12 molecular imaging system. An equivalent amount of PAK was found in both TritonX100-soluble (s) and TritonX100-insoluble (i) fractions. The C-PAK peptide, carried by the C-PAK$^+$ strain, was present in the TritonX100-insoluble fraction. The total amount of PAK remained unchanged in each strain. In the control cells, the total actin partitioned corresponded to 70% TritonX100-soluble and 30% TritonX100-insoluble fractions, whereas in the C-PAK$^+$ strain actin was at 10% in the TritonX100-insoluble and 90% in the TritonX100-soluble fractions.

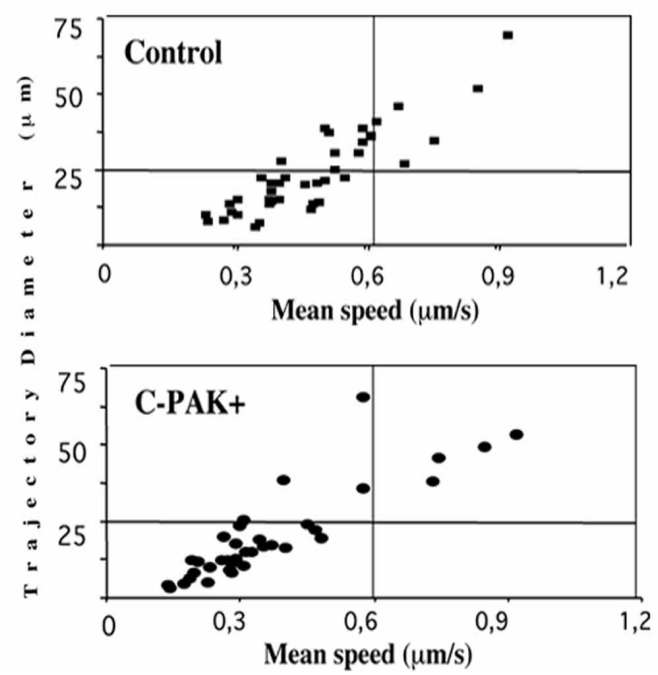

Fig. 6. Overproduction of C-PAK affects *E. histolytica* motility. The mean speed (μm/s) and the trajectory diameter (μm), representing the largest distance between two arbitrary points of the total trajectory of each amoeba (control ■ and C-PAK+ ●), were plotted. When the trajectory diameter was greater than the size of an amoeba (25 μm), we concluded that the parasite migrated, and when it was below 25 μm, the parasite was considered to be unable to migrate. Note that 80% of the analysed C-PAK+ population did not migrate. Velocities of amoeba from the control strain reached 0.9 μm/s. Each plot is a compilation of three distinct experiments.

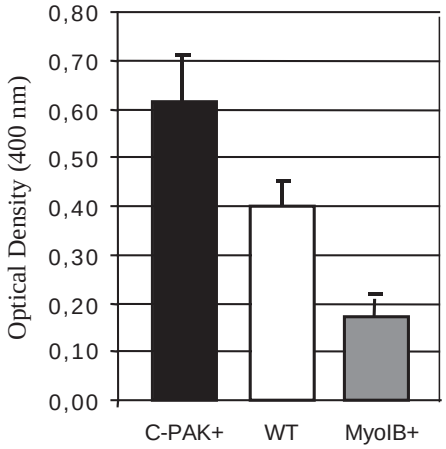

Fig. 7. Accumulation of C-PAK enhances phagocytosis of human RBCs by *E. histolytica*. The histogram shows the erythrophagocytosis rates of *E. histolytica* strains. Wild-type (WT) (white) and transfectants C-PAK (black) or MyoIB$^+$ (grey) were used. To quantify engulfed RBCs the concentration of haemoglobin inside parasites was measured as described previously (Voigt et al., 1999). Bars indicate standard deviations of the means after three independent experiments.

fraction were separated by SDS-PAGE. The partitioning of actin and EhPAK was revealed by anti-actin and anti-PAK, respectively. The enzyme was equally concentrated in the soluble and the insoluble fraction, suggesting that a fraction of EhPAK could be associated with the actin cytoskeleton and that there was no major change in this association in the transfectant strain. From this experiment, we also observed that in the C-PAK$^+$ strain, the C-terminal domain of EhPAK was partitioned in the insoluble Triton X100 fraction. The F-actin content in each cell fraction was also determined and quantified by western blot and phosphoimager analysis. The F-actin repartitioning indicated that in the *E. histolytica* control strain, as in other motile cells, the majority of actin was present in a TritonX100-soluble fraction (70% of total actin). In the C-PAK$^+$ strain, there was an increase in soluble actin of up to 90%, suggesting that the active form of EhPAK led to an increase in actin concentration in the TritonX100-soluble fraction. However, whether actin existed as short filaments or as globular forms needs to be determined. These observations were in agreement with the confocal microscopy analyses and confirmed that changes in EhPAK activity regulated the dynamics of the actin cytoskeleton in *E. histolytica*.

Effect of EhPAK on *E. histolytica* motility

We reasoned that the effects of EhPAK on actin dynamics and cellular morphology might result in alterations of *E. histolytica* motility. *E. histolytica* is a cell that aggregates and highly deforms, and in our experiments usual computer programs failed in tracking them individually. We therefore developed a procedure allowing the analysis of amoeba motility by combining video-microscopy and a customised image analysis programme developed 'in-house' (Materials and Methods) (Zimmer et al., 2002). Our computerised approach overcame the limitations of manual quantification, which was prone to user bias and was unreasonably time-consuming for large data sets as in this study and, of commercial software packages that consider isolated, well contrasted, non-deformable and non-aggregating cells. To determine whether EhPAK had a role in *E. histolytica* migration, control, PAK$^+$ and C-PAK$^+$ adherent trophozoites in culture flasks were observed by optical phase microscopy, video-recorded and computer analysed (Materials and Methods). To quantitatively measure cellular motility, we defined the 'trajectory diameter' as the largest distance between two arbitrary points of the trajectory. The size of an amoeba is roughly 25 μm, and by FACS analysis we verified that the control population and the C-PAK$^+$ strains had the same size (data not shown). We decided that an amoeba was migrating during the recorded time (5 minutes) when the interval between the two most distant points of its resulting trajectory was superior to 25 μm. Fig. 6 shows the plotted 'trajectory diameter' and the mean speed [trajectory length (μm)/time (s)] calculated for each amoeba of the two transfectant populations. The movement of an amoeba is influenced by its morphology, direction and speed; these different factors lead to heterogeneous behaviour of amoebae in the same culture during 5 minutes of recording. In the control population, 40% of the amoebae clearly displayed a trajectory diameter in excess of 25 μm, and in the C-PAK$^+$ population only 20% of amoebae had a displacement beyond 25 μm. The 'trajectory diameter' of C-PAK$^+$ cells and control cells were significantly different, as calculated by a Kolmogorov-Smirnov test which is more appropriate than a

chi-square test to analyse unbinned distributions (Press et al., 1992). The K-S statistics are D≈0.3, p≈0.04, indicating that the observed motility reduction of C-PAK$^+$ cells is significant. This result suggested strongly that overproduction of the activated catalytic domain of PAK inhibited *E. histolytica* migration and motility. The velocity and the trajectory of an *E. histolytica* strain overproducing the full-length PAK were measured but no significant change compared to the control strain was observed (data not shown).

Influence of EhPAK on the phagocytic activity of *E. histolytica*

In *E. histolytica*, motility and phagocytosis of human cells are two biological phenomena that require pseudopod extension. The fact that C-PAK overproducing amoeba were not motile and displayed multiple nascent but abortive pseudopodia prompted us to ask whether the phagocytosis pathogenic maker for *E. histolytica* was affected by EhPAK. To analyse the phagocytic activity, we incubated amoeba with human RBCs (10 minutes at 37°C with a ratio of 100 RBCs for 1 amoeba). The phagocytic efficiency was measured by spectroscopy as a function of haem content. We compared phagocytosis of C-PAK$^+$ transfectant amoeba with the control strain and a strain overexpressing myosin IB (MyoIB$^+$), which is known to be deficient in its phagocytic capacity (Voigt et al., 1999). We found a significant increase in erythrophagocytosis rate with C-PAK-overproducing amoeba (150%) compared with wild-type parasite (100%) and MyoIB$^+$ strains, which displayed a reduction in erythrophagocytosis of 50% confirming our previous data (Voigt et al., 1999). These results indicated that the lack of polarity of the C-PAK$^+$ strain did not inhibit phagocytosis and suggested that the phagocytic process of *E. histolytica* could be enhanced by the presence of multiple pseudopodia-like structures.

Discussion

The pathogenic amoeba *E. histolytica* possesses a complex signalling pathway leading to the motility required for tissue invasion. During its locomotion, rapid morphological changes are observed, suggesting that the actin cytoskeleton of this lower eukaryotic cell is highly dynamic. EhPAK, a putative p21-activated kinase, has been described in *E. histolytica*. This amoebic enzyme has no CRIB motif to bind to Rac and Cdc42. Nevertheless, our biochemical studies show that the N-terminal domain of EhPAK (150 amino acids) binds to activated Rac1 and not activated Cdc42. This is of relevance, since in *E. histolytica* small GTPases homologous to Rac have been reported but none to Cdc42. Two forms of Rac (RacA and RacG) have been described in *E. histolytica* as regulators of actin cytoskeleton organisation. The role of these Racs as regulators of EhPAK is still to be determined. However, EhRac could activate the enzymatic activity of EhPAK as it is described for eukaryotic PAKs where Rac (or Cdc42) binds to the PAK regulatory N-terminal domain and induces a conformational change that triggers PAK activation after relieving the kinase auto-inhibitory domain. The unfolding of PAK generates free access to the catalytic C-terminal domain and induces autophosphorylation at several sites of the protein, which increases its capacity to phosphorylate an exogenous substrate (Manser et al., 1994). The exception to this behaviour concerns PAK4, which differs from the other human PAKs because it selectively binds to Cdc42 but not to Rac, although it possesses a CRIB sequence and despite its apparent constitutive kinase activity. Binding to Cdc42 is necessary to recruit PAK4 to the Golgi where PAK4 can induce actin rearrangements (Abo et al., 1998). Likewise, an EhRac protein could regulate EhPAK by recruiting EhPAK to a defined cell compartment.

Overexpression of C-terminal domain of PAK causes a deregulation of pseudopod extension and inhibits *E. histolytica* migration

Constitutive overexpression of the DNA fragment encoding the C-terminal domain of EhPAK induces a dramatic morphological change with simultaneous formation of multiple membrane extensions and inhibition of cell polarisation. This result suggests that, when overproduced, C-PAK deregulates the endogenous enzyme EhPAK. By contrast, the overproduction of the full-length enzyme did not alter the wild-type phenotype, indicating that the twofold excess of the entire enzyme remains regulated probably by the function of the N-terminal domain. With the help of a dedicated image analysis program that takes into account the morphological changes of cells that move and deform rapidly. such as *E. histolytica*, we showed that C-PAK overexpression inhibited migration. These data indicate that the formation of a predominant polarised pseudopod is a regulated phenomenon that requires the full-length EhPAK. Our hypothesis is that the N-terminal domain of EhPAK could be a regulatory element that coordinates morphological changes in *E. histolytica* even in the absence of a CRIB motif. This phenotype is consistent with those encountered with PAKa from *D. discoideum*, where cells expressing only the kinase domain of PAKa present multiple membrane ruffles over the entire cell, demonstrating that the N-terminal part of PAKa is required for suppressing undesirable lateral pseudopod extensions (Chung and Firtel, 1999). In addition, the N-terminal part of mammalian PAK, through its different protein domains, is known to be involved in PAK cell localisation and to be necessary for the development of polarised lamellipodia.

Several proteins have been identified as PAK-binding partners. The adaptor protein Nck, known for its role in coupling activated receptor tyrosine kinase to various signaling pathways (Pawson et al., 1993) and the PIX/Cool proteins are required to target PAK to focal adhesion sites (Bagrodia et al., 1998; Bokoch et al., 1996; Manser et al., 1998; Oh et al., 1997). The fact that amoeba membrane extensions are formed and recycle rapidly, as well as the localisation of EhPAK to the pseudopod of migrating amoeba, would fit the model of de Curtis that links PAK to membranes of the pseudopod during mammalian cell migration, via a complex of Nck-PIX-paxillin-Rac (de Curtis, 2001). For instance, after induction of receptor capping in *E. histolytica*, EhPAK is recruited to the accumulated folded membrane that forms the uroid (Labruyère et al., 2000). The N-terminal domain of EhPAK does not possess a conventional SH3-binding motif necessary to bind Nck nor the conserved proline-rich PIX-binding sequence. Nevertheless, the N-terminal domain of EhPAK exhibits a proline-rich sequence that could interact with amoebic protein

partners controlling EhPAK localisation. In that sense, the N-terminus of EhPAK appears to have the minimal requirement to regulate pseudopod extension independently of CRIB or SH3 motifs. However, whether the activity of the N-terminal domain on cell polarisation depends on the PAK-Rac interaction via a parasite protein is under investigation. In addition, it will be interesting to evaluate whether the formation of new adhesion sites is altered in *E. histolytica* expressing the constitutive kinase, indicating a potential role for EhPAK in site adhesion formation.

Overexpression of a C-terminal domain of PAK enhances *E. histolytica* red blood cells phagocytic activity

Cortical actin polymerisation and the subsequent extension of pseudopodia are important components of the phagocytic mechanism of *E. histolytica* (Voigt and Guillén, 1999). Activation of the *E. histolytica* surface by the adhesion of RBCs leads to cytoskeleton rearrangements that are central to the process of phagocytosis. These rearrangements involve the action of small GTPases such as Rac A (Lohia and Samuelson, 1993) and the activity of the mechanoenzyme myosin IB (Voigt et al., 1999). In this work, we demonstrate that the increase in C-PAK in *E. histolytica* leads to the formation of multiple nascent pseudopod-like structures that concentrate EhPAK. Increase in the content of the catalytic domain of EhPAK has a major impact on parasite phagocytosis capacities since the C-PAK$^+$ strain displays a higher rate of RBC phagocytosis compared with the wild-type strain. This result raises the question of how pseudopodia extension is driven during the phagocytic process of *E. histolytica*. The phagocytic hyper-reactive phenotype of C-PAK$^+$ contrasts with the behaviour of *E. histolytica* strain overexpressing myosin IB, which has a reduced rate of phagocytosis and displays a normal motility and cell polarisation. Interestingly, among the substrates of PAK the regulatory light chain of myosin II (Chew et al., 1998), the myosin light chain kinase (Sanders et al., 1999) and the myosin IB heavy chain (Lee et al., 1996) have been described, suggesting a direct link between PAK and actin-myosin complexes. Two myosins have been reported in *E. histolytica*, the conventional myosin II and the non-conventional myosin IB. The last one was involved in phagocytosis (Voigt et al., 1999) and is similar to myosin IB from *D. discoideum* and *A. castellanii*, which are involved in pseudopodia formation. There is a striking difference between *E. histolytica* myosin IB and those from other amoebae: the heavy chain does not contain the serine/threonine amino acid at the usual PAK target phosphorylation site (Vargas et al., 1997a; Vargas et al., 1997b). Nevertheless, we do not know whether myosin IB in *E. histolytica* is the substrate of EhPAK even in the absence of a consensus phosphorylation site. *E. histolytica* also presents a conventional myosin II similar to those present in *A. castellanii*, *D. discoideum* and chicken smooth cells (Raymond-Denise et al., 1993). *E. histolytica* myosin II is involved in receptor capping, uroid formation and motility, and like EhPAK, is enriched at the rear of the parasite during these activities (Arhets et al., 1995; Arhets et al., 1998; Labruyère et al., 2000). The relationship between EhPAK and myosin II during capping of surface receptors is a matter under investigation.

In conclusion, our results of the analysis of the strain accumulating the C-terminal domain of EhPAK, C-PAK$^+$ strain, indicate that in *E. histolytica*, the protein kinase EhPAK is implicated in cell polarisation, motility and phagocytosis, processes dependent on actin cytoskeleton dynamics. A new challenge is to elucidate the nature of protein complexes associated with EhPAK, with the goal to determine key elements for the regulation of actin dynamics in the parasite. This will contribute to our understanding of the crucial steps for *E. histolytica* motility and phagocytosis that are necessary to invade and destroy human tissues during amoebiasis.

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