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The small GTP-binding protein RacG regulates uroid formation in the protozoan parasite *Entamoeba histolytica*

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SUMMARY

Entamoeba histolytica **is a protozoan parasite that invades human intestine leading to ulceration and destruction of tissue. Amoebic movement and phagocytosis of human cells is accompanied by characteristic changes in cell morphology. Amoebae become polarized, developing a frontal pseudopod and a well-defined rear zone of membrane accumulation designated the uroid. In motile eukaryotic cells, a phenomenon that contributes to movement is the capping of receptors at the cell surface. During the capping process, *E. histolytica* concentrates ligand-receptor complexes in the uroid. Interestingly, some of these surface receptors are involved in the survival of the parasite. While looking for regulators of capping and uroid formation, we identified RacG, an *E. histolytica* protein that is homologous to human Rac1. This protein belongs to the Rac subfamily of small GTPases implicated in interactions between the actin cytoskeleton and the membrane of mammalian cells. Cloning of the Eh*racG* gene and analysis of the protein activity either in murine fibroblasts or in *E. histolytica* revealed that EhRacG induces a characteristic Rac phenotype. When expressed in amoebae, an EhRacG-V12 mutant protein not only deregulated cell polarity, but also caused a defect in cytokinesis. Analysis of the cytoskeleton in amoebae bearing this mutant revealed that F-actin concentrated at the periphery of the cell. In addition, the number and localization of uroids were modified. These results suggest a role for EhRacG in amoebic morphogenesis and cytokinesis.**

Key words: Amoebiasis, Capping, Cytoskeleton, Entamoeba, RacG

INTRODUCTION

Entamoeba histolytica, a protozoan parasite, is the causative agent of amoebic dysentery. The virulence of this organism is derived from its capacity to invade the human intestinal tissue, to phagocytose erythrocytes and epithelial cells, and, eventually, to cause liver abscesses (Ravdin, 1995). Expression of these pathogenic activities requires a dynamic cytoskeleton that allows rapid changes in movement and morphology. During its migration on a matrix surface, *E. histolytica* acquires a spatial asymmetry characterized by a polar morphology. In the 'front' of cells, a pseudopod is formed, and in the 'rear', a uroid is generated by membrane folding (Bailey et al., 1992). Polarization of amoebae leads to both an extension of the plasma membrane which is necessary to form the pseudopod and the uroid, and a redistribution of F-actin which concentrates in these structures (Guillén, 1996). Other molecular events involved in migration, such as the rearward translocation of surface receptors by a capping process (Arhets et al., 1995), occur concurrently with the asymmetric distribution of F-actin. The capping process, particularly active in *E. histolytica*, probably participates in the adaptation of this parasite to its environment in tissues. Capping allows the accumulation of ligands, including complement components and anti-amoebae antibodies in the uroid and membrane shedding ensues (Hamelmann et al., 1993). By this process, *E. histolytica* extrudes bound ligands to the extracellular medium and, at the same time, eliminates a substantial membrane fraction without significant damage to the organism. This mechanism may be essential for escape from the host immune response.

During invasive amoebisis, *E. histolytica* penetrates intestinal tissue, kills the cells and interacts with the extracellular matrix (ECM). Amoeba-cell and amoeba-ECM interactions are mediated by particular surface receptors (Saffer and Petri, 1991; Vazquez-Prado and Meza, 1992). These interactions elicit amoebic responses, including protease secretion (Serrano et al., 1996), activation of signal transduction pathways, such as the adenylyl cyclase and protein kinase C pathways (Santiago et al., 1994; Vazquez et al., 1995) and rearrangement of the cytoskeleton (Bailey et al., 1985; Carbajal et al., 1995). In addition, recent data have demonstrated the role of the actomyosin complex in capping, uroid formation and membrane shedding (Espinosa-Cantellano and Martinez-Palomo, 1994; Arhets et al., 1995). How cytoskeletal activities are coordinated during these processes is

still under investigation. Studies conducted in a large variety of eukaryotic cell types have identified key signaling molecules that play a role in the regulation of cytoskeletal responses. Analysis of a subset of these signaling molecules, the small GTP-binding proteins from the Rho-family, has provided new insights into these process (Hall, 1994). Based on amino acid sequence comparison, the Rho family of GTPases encompasses three distinct subfamilies: Rho, Rac and Cdc42 (Hall, 1994). Representatives of these three subfamilies induce different types of cytoskeletal changes in response to signal transduction pathways triggered by growth factors or as a consequence of their overexpression. Rho controls the formation of stress fibers and focal adhesion plaques, Rac induces membrane ruffling, and Cdc42 induces formation of filopodia (Hall, 1994). The regulation of cytokinesis in various organisms is also believed to be controlled by members of this GTPase-family including Rho in the *Xenopus* embryo (Kishi et al., 1993), Cdc42 in HeLa cells (Dutartre et al., 1996), and RacE in *Dictyostelium discoideum* (Larochelle et al., 1996).

DNA sequence analysis has suggested that the *E. histolytica* genome encodes at least five small GTP-binding proteins belonging to the Rho-family. One of them is homologous to Rho1p and Rho4p of *Saccharomyces cerevisiae* (Lohia and Samuelson, 1993). The other four predicted proteins share similarity with human Rac (Lohia and Samuelson, 1996). Whether these proteins have distinct or overlapping functions is still an open question. In particular, their involvement in the regulation of cytoskeletal activities is not known. We describe here *racG*, a novel *rac* gene from *E. histolytica*. The encoded protein shares 85% similarity with human Rac1. We have established an amoebic cell line that expresses an activated form of RacG. In this work, we have observed that cytokinesis and the distribution of F-actin are impaired and that capping activity and uroid formation become unregulated in such cells. Our results, therefore, suggest that RacG of *E. histolytica* may be involved in cytoskeleton remodelling and in cytokinesis.

MATERIALS AND METHODS

Strains and culture conditions

The pathogenic strain of *Entamoeba histolytica* HMl:IMSS was grown axenically at 37°C in TYI-S-33 medium (Diamond et al., 1978). Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For recombinant DNA techniques, *Escherichia coli* DH5α was used. This bacterial strain was cultured in Luria-Bertani medium (Sambrook et al., 1989) in the presence of ampicillin at 50 μg per ml when required.

Cloning of the *racG* gene by PCR, inverse PCR, RACE and DNA sequence

The recombinant gene technology was completed according to protocols from the Molecular Cloning Laboratory Manual (Sambrook et al., 1989). PCR was originally performed to amplify fragments encoding myosin I; the primers used were deduced from consensus amino acid sequences for ATP binding sites (G X4 GKT) and for phosphorylation sites located on the heavy chain of myosin I (RDALAK). The primers were selected according to the codon usage of *E. histolytica* (Tannich and Hortsmann, 1992). The forward primer was 5′-GAATCAGGA/TGCTGGAAAA/GACT and the reverse primer was 5′-A/TGCTTTA/TGCA/TAA/GA/TGCATCTCT. PCR was done on genomic DNA (1 μg) during 40 cycles of amplification: 1 minute at 94°C, 10 seconds at 49°C and 2 minutes at 72°C with the Taq polymerase from Cetus, according to the manufacturer's recommendations. Amplified fragments were cloned into the M13 phage and their nucleotide sequence was determined by the dideoxy-chain terminator technique. One of the amplified fragments contained a sequence of the *racG* gene. The 3′ end of the gene was obtained by inverse PCR. To determine the DNA fragment to be amplified by inverse PCR, Southern blots were carried out on the amoebic genome using the first cloned DNA fragment as a probe. This genomic mapping revealed an *Ase*I fragment of 1 kb containing a portion of the known sequence. Then, 4 μg of genomic DNA were digested by *Ase*I, and 10 ng of the digested DNA was self-ligated using the T_4 DNA ligase enzyme. The ligation mixture was directly used as a template for PCR using the following primers: 5′-TCAGTTGAT-GGATATGATAAT and 5′-ATGTACCAATTATTGTTGTTG. Thirty cycles of 1 minute at 94°C, 1 minute at 49°C and 1 minute at 72°C were completed in the presence of the Taq polymerase from Cetus according to the manufacturer's directions. The amplified fragment of 1 kb was directly sequenced by the dideoxy chain terminator technique. To obtain the sequence information from the 5′ end of the *racG* gene, we used the rapid amplification of cDNA ends (RACE) technique (Bertling et al., 1993) (Clontech Ampli Finder RACE Kit) and the following primers 5′-TCAGTTGATGGATATGATAAT and 5′-CTGTTGGAATATATTCATTTG. RNA was purified from exponentially growing amoebae (Vargas et al., 1996). The amplified cDNA fragment of 200 bp was directly sequenced by the dideoxy chain terminator technique. Finally, the entire *racG* gene was obtained by PCR amplification on the λ ZAP cDNA (Kohler and Tannich, 1993), with the forward primer 5′-CCTATGAGACCAGTGAAACTT and the reverse primer 5′-AACAACTTCTTCAATTTATCA which allowed the direct cloning of the amplified DNA into the pGEX-2KT expresion vector (Pharmacia). This vector was digested with *Eco*RI endonuclease and filled in with Klenow fragment of the DNA polymerase to produce blunt ends for the ligation. Recombinant plasmids were selected after transformation in *E. coli* DH5α by colony hybridization using the *racG*-M13 cloned fragment as a probe. Two positive clones were selected, plasmid DNA was prepared, and the nucleotide sequence was determined. All nucleotide sequences were analyzed by the GCG computer program.

ADP-ribosylation assay

To test the ADP-ribosylation of EhRacG, we used previously published protocols (Rubin et al., 1988). In short, 300 ng of purified human RhoC or amoebic RacG were incubated with 130 ng of purified C3 transferase in the presence of 0.1% BSA and ^{32}P-NAD (30 Ci mM, Dupont NEN). The following incubation buffer was used: 2 mM AMP, 1 mM $MgCl_2$ in 20 mM Hepes pH 8. The proteins were then resolved in SDS-12.5% PAGE and the gel was subsequently autoradiographed. The autoradiogram was scanned using a Microtek Scanmaker E3.

Construction of the mutant allele *racG*-V12

Starting with single stranded DNA from the wild-type gene, the primers carrying mismatches at the nucleotides necessary for the codon change were hybridized on the two DNA strands. PCR was performed to amplify fragments incorporating these mismatches. A 50 bp fragment corresponding to the 5′ end of the gene was amplified with oligonucleotides 5′-GACGGCAACATCACCGAC (reverse primer containing the mutation) and 5′-CCCGGGAATTCCTATGAGA-CCAGTG (forward primer) which incorporated a *Eco*RI site at the 5′ extremity of this fragment. The fragment of 650 bp containing most of the gene including the 3′ end was amplified with the oligonucleotides 5′-GTCGGTGATGTTGCCGTC (forward primer containing the mutation) and 5′-GTGAGAATTCAAATTAGAAT-AATGAGCATCC (reverse primer) which incorporated an *Eco*RI site at the 3′ extremity. PCR reactions were done on 500 ng of genomic

DNA with Vent DNA polymerase (Biolabs). After PCR, these fragments were denatured and hybridized (ratio 1:1). Double stranded DNA was synthesized with the Vent DNA polymerase following the manufacturer's directions (Biolabs). The entire gene was amplified by PCR, using reverse and forward primers located at the extremities of the gene. The amplified gene was purified, digested by *Eco*RI, and cloned in frame with the gene encoding GST. The recombinant plasmids were detected by colony hybridization with a ^{32}P-labelled primer, internal to the *racG* gene. Two recombinant plasmids were purified and the nucleotide sequence of the insert was determined. Both plasmids showed that the codon exchange leading to Val12 was introduced at the desired position and that the mutated allele of *racG* was in frame with the gene encoding GST. In addition, the entire gene DNA sequence was verified, confirming that no undesired mismatches were introduced during these PCR reactions.

Expression and purification of GST-EhRac, GST-EhRac-V12 and GST-HsRac1-V12 and microinjection assay

Expression and purification of GST and GST recombinant proteins, EhRac, EhRac-V12 and Hs Rac1-V12 was conducted according to published methods (Ridley et al., 1992). GST-EhRac1 was eluted from glutathione beads (according to Pharmacia protocols), dialysed against 20 mM Hepes buffer, and then used in an ADP-ribosylation assay. Purified EhRac-V12 (300 μg/ml) and Hs Rac1-V12 (300 μg/ml) were used in microinjection. Culturing of Swiss 3T3 cells and microinjection were done according to established conditions (Nobes and Hall, 1995) without inhibitors. Active protein concentrations were determined by the filter binding assay using 1 μM of protein loaded with 10 μM of ^{35}S-GTPγS (1,000 cpm/pmol) following conditions previously described (Self and Hall, 1995); roughly, preparations of EhRacG-V12 and Hs Rac1-V12 were 50% active. Proteins were microinjected along with a marker protein (mouse immunoglobulins at 0.5 mg/ml). Thirty minutes after microinjection, the cells were fixed with 3.7% paraformaldehyde for 30 minutes. Coverslips were incubated in PBS containing 0.1% Triton X-100, washed with 50 mM NH_4Cl in PBS and blocked with 1% BSA-PBS for 30 minutes. Injected cells were localized by staining for 20 minutes with a rhodamine-conjugated anti-mouse IgG and F-actin was stained with FITC-phalloidin (Molecular Probes). Coverslips were mounted on a glass slide with DABCO-PBS. Fluorescent samples were examined on a Leica DIAPLAN confocal laser scanning microscope (CLSM). Rhodamine-labeled samples were visualized with a high pass RG590 (after excitation at 568 nm). To visualize fluorescein, we used a narrow band interferential filter centered on 535 nm (excitation at 488 nm). Observations were performed in ten planes from the bottom to the top of each cell. The distance between scanning planes was 0.5 μm. Images of serial confocal sections were analyzed with the CLSM-Leica software. Photographs were taken on Kodak T-max 400 film with a 35 mm camera mounted on a Polaroid Freeze-Frame video recorder monitor.

Production of antibodies against an internal peptide from EhRacG

Taking into account the differences in the amino acid sequence of the EhRacG when compared with HsRac1, a peptide of 15 amino acid 'EQVKRLAEKNIVPIQ' was synthesized. This peptide was coupled to BSA (Zegers et al., 1990) for immunizations. Preimmune serum was obtained from two New Zealand white rabbits before immunization. The immunization schedule was comprised of four injections, each with 100 μg of protein, at 15-day intervals. The first injection was intradermic in the presence of an equal volume of incomplete Freund's adjuvant, the second was subcutaneous and the others were intramuscular without adjuvant. Fourteen days after the last immunization, the immune serum was collected. The presence of Ig against the RacG peptide was tested by ELISA and by immunoblot analysis against the GST-RacG hybrid protein. Immunoglobulins from the immune serum were purified by Protein A-Sepharose chromatography (Pharmacia) and frozen at −70°C until further use.

Cloning of RacG-V_{12} into ExEhneo vector and transfection into parasites

The pExEhNEO vector is a derivative of pEhNEO/CAT (Hamann et al., 1995) in which the choramphenicol cassette was deleted and replaced by a multicloning site containing *Kpn*I, *Sma*I and *Bam*HI restriction sites (E. Tannich, unpublished data). pEhNEO/CAT vector alone was used as a control in transfection experiments. The *racG*-V12 gene was excised by *Eco*RI digestion from the pGEX-2KT plasmid, DNA extremities were filled in by the Klenow enzyme and this DNA was ligated into the ExEhNEO vector digested with *Sma*I. One of the recombinant plasmids was purified and the cloning junctions were verified by nucleotide sequencing. Two hundred micrograms of purified recombinant plasmid or vector alone were electroporated into growing *E. histolytica* (Hamann et al., 1995). Drug selection was started 48 hours after transfection, using G418 at 10 μg/ml. After 48 hours of selection in the presence of 10 μg/ml of G418, the drug concentration was diminished to 3 μg/ml in both transfectant culture, RacG-V12 or NEO/CAT. Under such conditions the non-transfected wild-type strain cannot survive.

Induction of the capping of surface receptors and confocal laser scanning microscopy

Activation of parasites for uroid formation and capping of surface receptors was performed by incubation of growing amoebae in the presence of Concanavalin A (Arhets et al., 1995). After ConA treatment, the amoebae were fixed with 3.7% paraformaldehyde for 30 minutes and permeabilized with 0.25% Triton X-100 for 30 seconds. The cells were then centrifuged at 700 ***g*** for 5 minutes, incubated again in 3.7% paraformaldehyde for 30 minutes, washed in PBS, and incubated in PBS containing 50 mM NH_4Cl for 30 minutes. Cells were incubated for 30 minutes in PBS containing 1% of bovine serum albumin (BSA). For the labeling of EhRacG and myosin II, fixed trophozoites were incubated for 1 hour with diluted (1/20) anti-EhRacG purified serum or (1/20) anti-myosin II purified serum (Rahim et al., 1993) in PBS-1% BSA for 30 minutes, and then for 30 minutes in a 1:200 dilution of rhodamine-labeled goat anti-mouse immunoglobulin (Sigma) preabsorbed on trophozoites. Preabsorption with trophozoites was performed as previously described (Rahim et al., 1993). The preparations were further incubated in PBS containing 1% BSA for 30 minutes. at room temperature, briefly washed in PBS, and mounted on a glass slide with DABCO-PBS. For F-actin and some RacG labeling experiments, amoebae were used without stimulation with ConA. F-actin was stained with FITC- or rhodamine-conjugated phalloidin (Bodipy, Molecular Probes). Nuclei were stained for DNA with propidium iodide purchased from Molecular Probes. Fluorescent or immunofluorescent samples were examined by CLSM as described above. Observations were performed in fifteen planes from the bottom to the top of each cell. The distance between scanning planes was 0.5 μm.

Transmission electron microscopy (TEM)

10^7 growing amoebae were harvested by centrifugation at 700 ***g*** for 10 minutes, washed in PBS and then resuspended in 0.5 ml of PBS. Cells were fixed 1 hour at 4°C in a mixture of 1.6% glutaraldehyde and 1% tannic acid in 0.1 M phosphate buffer at pH 7.4 and then washed three times in the same buffer. Cells were then pelleted and resuspended in the same buffer containing 15% BSA. The supernatant was decanted and 10 μl of 25% glutaraldehyde was added. The solidified pellet was post-fixed for 1 hour at 4°C with 1% OsO_4 in the same buffer. The pellet was rinsed in distilled water, dehydrated in an ethanol series, and embedded in epoxy resin. Thin sections were prepared on an LKB Nova microtome (Leica) and observed in a JEOL 1010 electron microscope operating at 80 kV.

RESULTS

Identification of the RacG encoding gene

PCR reactions using the *E. histolytica* genome as a template allowed the identification of genes encoding proteins with consensus sequences for ATP or GTP binding sites. Computer analysis of one of the amplified DNA fragments showed an open reading frame of 118 amino acids that shares 88% similarity with human Rac1 protein (HsRac). To obtain the entire gene, inverse PCR was performed on DNA to amplify the 3′ end. In addition, RACE was used on RNA to amplify the 5′ end. The nucleotide sequence of both amplified fragments was determined. Consequently, the completed sequence data enabled PCR amplification of the entire gene from a cDNA library. We succeeded with this PCR amplification, a result which indicates that the cloned gene is expressed in *E. histolytica*. Analysis of the nucleotide sequence by the GCG computer program revealed a 199 amino acid ORF (Fig. 1A). Using the data base of the National Center for Biotechnology Information (NCBI), a search for protein homologies indicated that the encoded protein was closely related to small GTPases belonging to the Rho family, Rac subfamily (Fig. 1A). Specifically, 85% similarity was found with both Rac1 and Rac2 identified in humans, 82% with Rac1 from *Drosophila melanogaster* and 81% with EhRacA.

The sequence of four genes and two pseudogenes that predicted *E. histolytica* proteins homologous to Rac has already been described (Lohia and Samuelson, 1996). Since the gene described here appears to be different from the other Eh*rac* genes, we named it *racG*, following the nomenclature of the different Eh*rac* genes. Alignment of the EhRacG protein sequence with other members of the EhRac subfamily (Fig. 1B) permitted sequence comparison of EhRac proteins and demonstrated conservation of the four GTP-binding regions that are also found in all other small GTP-binding proteins. This indicated that EhRacG might present the same GTPase activity described for Rac GTPases (Gilman, 1987). The GTP-binding regions in EhRacG are the phosphate binding region GDGAVGKT (amino acids 10-17), the region that interacts with the gamma phosphate WDTAGQE (amino acids 56-62), the guanine specificity region TKID (amino acids 115-118), and the conserved SAL sequence (amino acids 158-160). A characteristic prenylation motif of Rac proteins is present in the carboxyl-terminal domain of EhRacG at Cys_{196}. Small differences were noted between amino acids 38 to 48 in which the classical FENY motif of Rho proteins was found. The only Rac protein known so far to present a similar motif is the recently described RacE from *D. discoideum* (Larochelle et al., 1996). The protein sequence information obtained by this analysis indicates that EhRacG presents extensive similarity to Rac.

ADP ribosylation of RacG by the C3 exoenzyme

The FENY motif of the EhRacG protein is also found in the segment of Rho proteins targeted by the *Clostridium botulinum* C3 exoenzyme (Sekine et al., 1989). The C3 exoenzyme, a transferase, splits NAD into ADP-ribose and nicotinamide and transfers the ADP-ribose moiety to Rho, leading to inhibition of the GTPase activity. C3 transferase has been used to investigate Rho-dependent signaling pathways as other members of the Rho-family essentially do not serve as substrates for ADP-ribosylation. Since EhRacG contains the FENY motif, we decided to determine whether or not this protein is a target for C3 transferase mediated ADP-

A

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          1
Hsrac1    MQAIKCVVVG DGAVGKTCLL ISYTTNAFPG EYIPTVFDNY SANVMVDGKP
Hsrac2    MQAIKCVVVG DGAVGKTCLL ISYTTNAFPG EYIPTVFDNY SANVMVDSKP
 Drac1    MQAIKCVVVG DGAVGKTCLL ISYTTNAFPG EYIPTVFDNY SANVMVDAKP
EhracA    MQAVKCVIVG DGAVGKTCLL ISYTTNAFPN EYIPTVFDNY SATVMVDSKP
EhracG    MRPVKLVIVG DGAVGKTCML ISYTTNAFPN EYIPTVFENY NSSLVVDDVK

          51
Hsrac1    VNLGLWDTAG QEDYDRLRPL SYPQTDVFLI CFSLVSPASF ENVRAKWYPE
Hsrac2    VNLGLWDTAG QEDYDRLRPL SYPQTDVFLI CFSLVSPASY ENVRAKWFPE
 Drac1    INLGLWDTAG QEDYDRLRPL SYPQTDVFLI CFSLVNPASF ENVRAKWYPE
EhracA    INLGLWDTAG QEDYDRLRPL SYPQTDVFLI CFSVVSPPSF DNVSSKWQPE
EhracG    INLGLWDTAG QEDYDRLRPL SYPSTDVFLV CFSVIAPASY ENVEGKWKPE

          101
Hsrac1    VRHHCPNTPI ILVGTKLDLR DDKDTIEKLK EKKLTPITYP QGLAMAKEIG
Hsrac2    VRHHCPSTPI ILVGTKLDLR DDKDTIEKLK EKKLAPITYP QGLALAKEID
 Drac1    VRHHCPSTPI ILVGTKLDLR DDKNTIEKLR DKKLVPITYP QGLAMAKEIG
EhracA    VSHHCPKTPC LLVGTKLDMR EDKEQLKRLE EKKITPITTE QGEAKCKDIG
EhracG    IDQHCPNVPI ILVGTKIDIR DDPEQVKRLA EKNIVPIQPP QGDELAKKIG

          151
Hsrac1    AVKYLECSAL TQRGLKTVFD EAIRAVLCPP ...PV....K KRKRKCLLL.
Hsrac2    SVKYLECSAL TQRGLKTVFD EAIRAVLCPQ ...PT....R QQKRACSLL.
 Drac1    AVKYLECSAL TQKGLKTVFD EAIRSVLCPV ...LQ....P KSKRKCALL
EhracA    AVKYIECSAL TQKNLRLVFD EAVRAVISPA ...GGAKKDK KNNRGCLLF.
EhracG    AVKYIECSAL TQANLKLVFE EAVRAVLAKA AKEPTGKKEK GGKKGCSLF.
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B

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          1
EhracA    .....MQAVK CVIVGDGAVG KTCLLISYTT NAFPNEYIPT VFDNYSATVM
EhracB    .......... ....GAGGVG KTCLLVSYTT NAFPTEYVPT VFDNYSATVM
EhracG    .....MRPVK LVIVGDGAVG KTCMLISYTT NAFPNEYIPT VFENYNSSLV
EhracD    AAPTDAKSVK LVVVGDGSVG KTCLLICYTT NEFPKDYVPT VFDNYMAPMT
EhracC    .MSEKPTSIK LVVVGDGAVG KTCLLICYTT NEFPKDYIPT VFDNYVVSLT


          51
EhracA    VDSKPINLGL WDTAGQEDYD RLRPLSYPQT DVFLICFSVV SPPSFDNVSS
EhracB    VDSRPINLGL WDTAGQEDYD RIRPLSYPQT DVFLLCFSVV SPPSFENISS
EhracG    VDDVKINLGL WDTAGQEDYD RLRPLSYPST DVFLVCFSVI APASYENVEG
EhracD    VDGEPINLGL WDTAGQEDSE QLRPLSYPNT DLFLLCFSVI SRTSFNNISS
EhracC    AGTRQIQLAL WDTAGQEEYD QLRPLSYSSA SIFLICFSVT SSVSYDNVIT


          101
EhracA    KWQPEVSHHC PKTPCLLVGT KLDMREDKEQ LKRLEEKKIT PITTEQGEAK
EhracB    KWKPEVSHHC PNAPCLLIGT KIDIRDEQTQ TNKTCDKKIE PITSEQGEAK
EhracG    KWKPEIDQHC PNVPIILVGT KIDIRDDPEQ VKRLAEKNIV PIQPPQGDEL
EhracD    KWLPEIKHYE PKCKMMVVGT NTDCRNDEAM IRKLADENQK PITTEEGEKL
EhracC    KWHPEVIHFA PKVPIILVGT KLDTRNDPAI VKRLTEQGMT VINTAKGEEL


          151
EhracA    CKDIGAVKYI ECSALTQKNL RLVFDEAVRA VISPAG...G AKKDKKNNRG
EhracB    CKDIGALKYI ECSALTQKNL RYVFDEA... .......... ..........
EhracG    AKKIGAVKYI ECSALTQANL KLVFEEAVRA VLAKAAKEPT GKKEKGGKKG
EhracD    AKDIKAICYM ECSALTRSGL NQVFDEAIHI VLNK...... NQSSKKSSKK
EhracC    KNRIKAVKYI ECSAKTSENL KTVFDEAVKT VL........ .MNKPQQRSK


          201
EhracA    CLLF
EhracB    ....
EhracG    CSLF
EhracD    CALL
EhracC    CALL
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Fig. 1. Alignments of EhRacG with other proteins. (A) Searching for homologues of EhRacG in NCBI produced high scores of similarities with HsRac1 (GenBank accession number P15154), HsRac2 (GenBank accession number P15153), Drac1 (GenBank accession number U11823) and EhRacA (GenBank accession number U29720). Amino acid alignments were produced using the GCG computer program and the pileup function. (B) Alignment of EhRac proteins (Lohia and Samuelson, 1996) and this contribution was done by the same method. The Eh*racG* gene nucleotide sequence has been registered with the GenBank accession number AF055340.

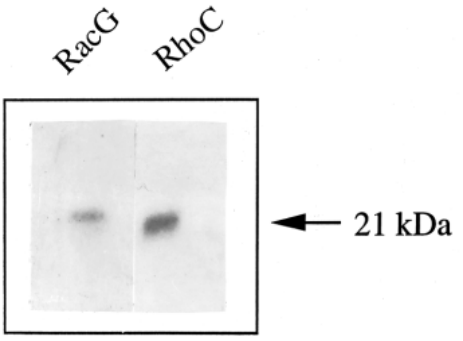

Fig. 2. ADP-ribosylation of EhRacG by *Clostridium* ADP-ribosyltransferase C3. Purified RacG (lane 1) or RhoC (lane 2) proteins were ADP-ribosylated with 0.1 μg of C3. Samples were subjected to SDS PAGE and ^{32}P-ADP-ribosylated proteins were analyzed by autoradiography.

ribosylation. EhRacG was produced in *E. coli* as a hybrid GST-RacG protein. The RacG moiety was obtained after digestion with thrombin and equivalent quantities of HsRhoC and EhRacG were used as substrates for ADP-ribosylation with purified C3 transferase. After the modification reaction, the proteins were analyzed by SDS-PAGE and the incorporated radioactivity detected (Fig. 2). Both proteins were ADP-ribosylated in this assay. However, ADP-ribosylation of human RhoC (HsRhoC) appeared more intense. The ratio of ADP-ribosylation obtained by the comparison of the radioactivity within the two bands was 1:16. These results indicate that there is more ADP rybosylation of the HsRhoC protein. A previous report indicates that differences of 20 times can be observed when ADP-ribosylation of HsRhoA versus ADP-ribosylation of HsRac1 are compared (Ménard et al., 1992). This 20-fold difference was considered significant and, consequently, the authors concluded that HsRac1 is not modified by C3 exoenzyme. Consequently, in the case of EhRacG, the low level of ADP-ribosylation we observed most likely indicates that its FENY motif is not sufficient to confer a high sensitivity to the C3 exoenzyme.

Construction of the RacG-V12 mutant and characterization of the phenotype in mice Swiss 3T3 fibroblasts

To determine whether the EhRacG protein exhibits a Rac phenotype, its capacity to induce cytoskeletal changes in mice fibroblasts was tested. For this purpose, a constitutively active form of RacG was constructed by changing amino acid 12 from Gly to Val, equivalent to the oncogenic mutation in ras (Hall, 1994). The GST-RacG-V12 hybrid protein was purified from bacteria. The Rac-V12 moiety was obtained after digestion with thrombin and then microinjected into the mouse Swiss 3T3 cell line, a system classically utilized for phenotypic studies of small GTPases (Ridley et al., 1992). To detect the microinjected cells, mouse IgG mixed with the purified Rac protein was used as a tracer. Microinjection of the IgG alone had no effect on actin organization and was only used for the visualization of microinjected cells. As the control, the human Rac1-V12 protein was also purified and microinjected into the cells. After microinjection, the localization of polymerized actin was observed by staining with FITC-phalloidin.

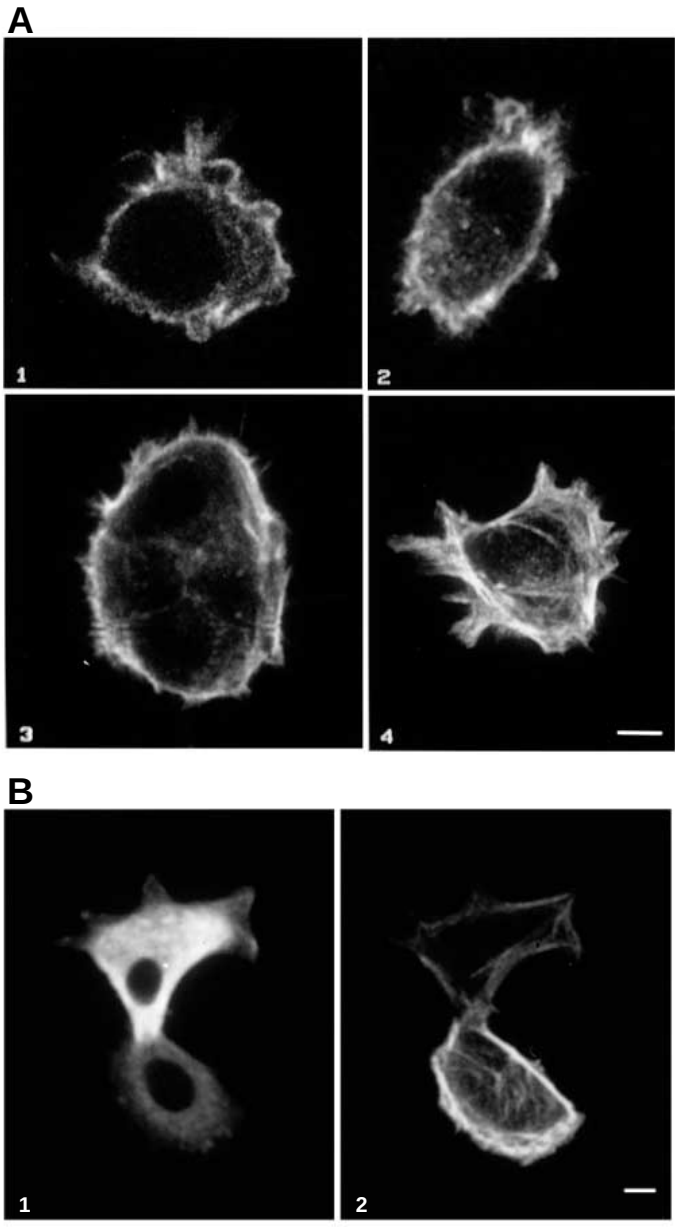

Fig. 3. Actin reorganization induced by microinjection of HsRac1-V12 or EhRacG-V12 in mice fibroblasts. (A) Serum-starved Swiss 3T3 fibroblasts microinjected with EhRacG-V12 (1 and 2), HsRac1-V12 (3) and mouse IgG (4) were fixed and stained with FITC-labeled phalloidin and TRITC-labeled anti-mouse IgG. The fluorescence micrographs show that stress fibers present in control fibroblasts disappear in cells microinjected with RacV12 proteins. Actin relocalization under the plasma membrane and the appearance of ruffles can be seen in microinjected cells. (B1) Micrograph corresponding to fluorescence of IgG which was mixed with EhRacG-V12 for microinjection. Fluorescence indicates that the upper cell was microinjected and that the lower cell was not microinjected. (B2) F-actin was stained with FITC-labeled phalloidin which detects actin filaments. Diminution of stress fibers is observed in the microinjected cell. Labeling was analyzed by CLSM. The focal plane number 3 from the bottom of each cells is shown. Bar, 5 μm.

Microinjection of HsRac1 or EhRacG caused a similar alteration in the actin cytoskeleton (Fig. 3). Fibroblast stress

Table 1. Microinjection of Rac-V12 proteins into mice fibroblast Swiss 3T3 strain

Protein source	Exp no.	No. cells	Microinjection	Rounded	%Rac phenotype
Amoeba	1	117	35 (29.9%)	11	68.5
Rac G-V12	2	133	34 (26.5%)	10	70.5
	3	119	41 (34.4%)	10	75.6
	4	132	51 (59.6%)	11	80
Human	1	111	23 (20.7%)	7	70
Rac 1-V12	2	106	32 (31%)	9	71.8
Mouse IgG	1	124	39 (31.4%)	0	0
	2	199	55 (28.6%)	9	0

fibers disappeared and polymerized actin mainly accumulated under the plasma membrane which was undergoing ruffling activity; this phenotype is classically associated with Rac protein (Ridley et al., 1992). The phenotypic analysis also showed that some of the microinjected cells rounded up. These rounded cells still attached to the culture flask and displayed labeling for F-actin at the membrane subcortical level. To quantitate cells expressing the Rac phenotype among the microinjected cells, a scoring system was used in which rounded cells were substracted (Table 1). Using this criterion, we determined that about 70% of the cells microinjected with either EhRacG or HsRac1 displayed the Rac phenotype. These results indicate that EhRacG, which is 85% homologous to HsRac1, elicits cytoskeletal rearrangements similar to those induced by human Rac1 in the Swiss 3T3 cell line.

Cellular localization of EhRacG in amoeba

To localize EhRacG in amoeba, a specific serum was raised against a peptide comprising amino acids 124 to 138. This antibody detected a protein of 21 kDa in the total amoebic protein extract. To investigate whether or not RacG is activated during capping of surface receptors, growing amoebae were incubated with fluorescent ConA. They were then fixed and treated for immunofluorescence staining with the anti-RacG serum. The relative distribution of RacG and ConA was analyzed using immunolabeling and fluorescence. When treated in the presence of ConA, 60% of the amoebae formed caps containing ConA-receptor complexes whereas the remaining 40% were considered as resting amoebae. In these last cells, RacG labeling appeared diffuse in the cytoplasm and no specific structure was observed (Fig. 4). When amoebae undergo capping, the plasma membrane is observed to fold towards the rear part of the cell and a uroid is subsequentally formed. In addition, cytoskeletal rearrangements also occur during this process (Arhets et al., 1995). Analysis of the parasites undergoing the capping process showed polarized amoebae with one uroid in which FITC-labelled ConA was concentrated (Fig. 4). Staining of these amoebae with the anti-RacG polyclonal antibody showed a diffuse labelling of the cytoplasm as well as the enrichment of EhRacG in the uroid region. This labeling was observed in all amoebae undergoing capping and was not detected when the preimmune serum was used instead of the anti-RacG serum.

Cloning of *racG* into amoebae, specific expression vector and analysis of the transfectant parasites

E. histolytica is a eukaryotic cell with a very versatile

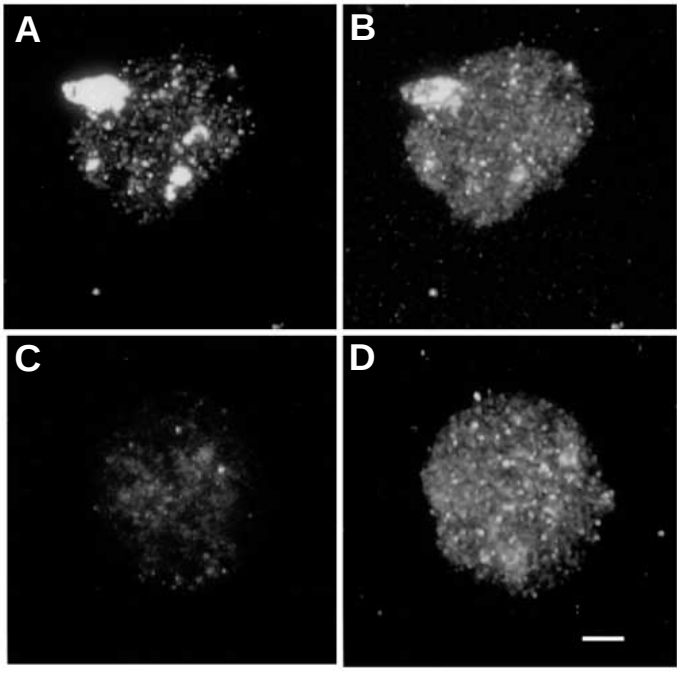

Fig. 4. Distribution of EhRacG in *E. histolytica* incubated with Con A to induce capping. The cells were incubated with FITC-labeled ConA for 10 minutes at 37°C, fixed, processed for immunofluorescence staining with an anti-EhRacG antibody, and analyzed by CLSM. The micrograph shows the distribution of ConA and EhRacG in both resting (C,D) and capping amoeba (A,B). RacG is diffusely distributed throughout the cytoplasm in resting amoebae whereas a significant portion of the molecule appears concentrated in the uroid when capping is induced (B,D). ConA is concentrated in the cap formed at the uroid (A,C). Both images correspond to the reconstitution of 10 focal planes from the same amoeba. Bar, 5 μM.

cytoskeleton which, in conjunction with other factors, forms a frontal pseudopod that is essential for movement. Stress fibers cannot be detected in this cell (Guillén, 1996). Thus, methods such as the microinjection of molecules cannot be used with these amoeboid cells since they do not survive. An alternative methodology used to introduce foreign molecules into *E. histolytica* is electroporation. To evaluate the role of EhRacG in amoebic cytoskeletal functions, the protein was overexpressed in amoebae. The gene encoding the dominant active RacG-V12 protein was transfected into *E. histolytica*. Transfectant cells containing either the vector alone or the *racG*-V12 recombinant plasmid were isolated in the presence of G418. To enhance the survival of transfectants containing the *racG*-V12 gene, the dose of G418 was diminished to 3 μg/ml after selection at 10 μg/ml. This allowed the recovery of the *racG*-transfected amoebae since high doses of G418 were lethal to these transfectants.

To analyze stable phenotypes in the transfectant population, we attempted to clone these cells but were unable to do so as *E. histolytica* transfectants, unfortunately, do not survive cellular cloning. Thus, we were forced to examine the phenotypic changes accompanying overexpression of RacG-V12 in a nonclonal population and heterogeneous phenotypes were detected. The different phenotypes may reflect the variability of the plasmid copy number in each transfectant.

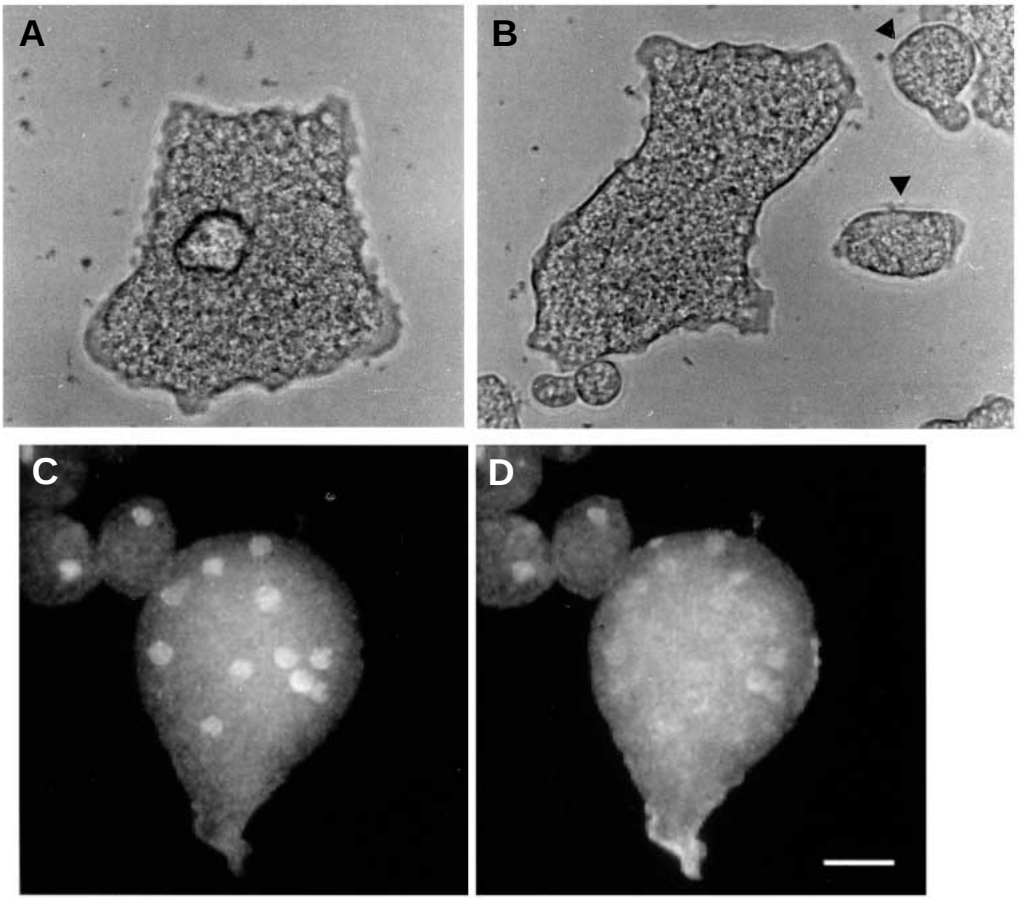

Fig. 5. Cytokinesis defect of *E. histolytica* transfected with *EhracG*-V12 mutant gene. After selection of transfectant amoebae, the parasites were grown in 3 μg/ml of G418. (A,B) Amoebae were visualized by phase contrast microscopy; several giant cells are shown. Compare to the 20 μm regular-sized amoebae (B, arrowheads). (C,D) Amoebae were fixed and strained for DNA or F-actin. (C) A reconstitution of focal planes observed when amoeba were stained for DNA. (D) A reconstitution of focal planes analyzed for distribution of F-actin in the same cells. In the giant amoebae, 10 nuclei are visible as compared to only one nucleus in the regular-sized cell. In addition, F-actin appears mainly diffuse in the cytoplasm of giant cells. Bar, 20 μm.

The most striking phenotype observed was seen in approximately 10% of the population. This phenotype was characterized as giant cells displaying impaired cytokinesis since all these giant cells were multinucleated. This phenotype was leaky as it was observed only during the first passages of the transfectants. These giant amoebae, when compared with the other transfectants, were not motile and appeared flat (Fig. 5A). This striking phenotype was never observed in cells transfected with the NEO/CAT vector plasmid alone. Although the population of giants cell was very difficult to manipulate, the cells were fixed and the localization of polymerized actin was observed by fluorescence. The visualization by CLSM of FITC-labeled phalloidin showed that F-actin was essentially dispersed throughout the cytoplasm. In addition, these cells were multinucleated (Fig. 5B) as revealed by staining with propidium iodide. The rest of the transfectant population consisted of amoebae that were similar to control cells in size (20 μm), in growth rate (8 hours generation time), and in cytokinesis. Further analysis was conducted using these regular sized amoebae which represented 90% of the transfected population. The expression of RacG was assessed by immunoblots. Proteins from EhRacG-V12 transfectants and from amoebae transfected with the vector alone were analyzed (data non shown). The results showed that there is 1.3 times more RacG in the transfectant population as compared to the control culture. This observation suggests that the phenotypical changes observed in the RacG-V12 expressing cells is mostly due to the presence of this constitutively active protein since there was not a significant difference in the quantity of the EhRacG protein expressed in each transformant cell line. The distribution of F-actin by FITC-phalloidin labeling in these transfectants was then examined and compared with the distrution of F-actin in control cells. A normal F-actin redistribution was found in NEO/CAT transfected control cells (Fig. 6A): filamentous-actin was concentrated in phagocytic

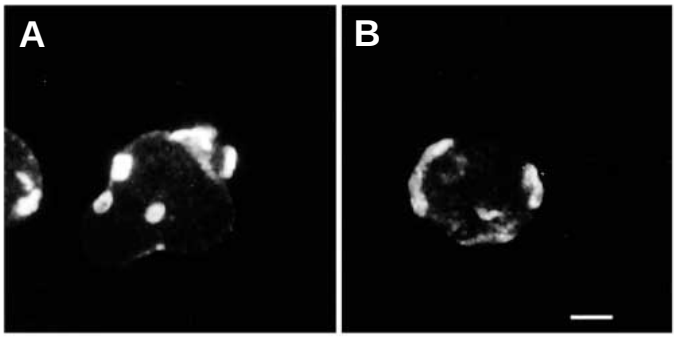

Fig. 6. Distribution of F-actin in *E. histolytica* transfected with the pNEO/CAT or with the Eh*racG*-V12 mutant gene. Transfectant amoebae were fixed and stained for F-actin using FITC-conjugated phalloidin. CLSM was performed and fifteen focal planes were analyzed, reconstitution of images is shown in the micrograph. F-actin in pNEO/CAT transfect cells accumulates in adhesion plates and in phagocytic stroma (A). F-actin in Eh*racG*-V12 transfectant accumulates under the plasma membrane (B). Bar, 10 μM.

stomas and in the focal adhesion plates. By contrast, in the RacG-V12 expressing cells, F-actin was found concentrated in cortical areas close to the plasma membrane (Fig. 6B), indicating massive redistribution, a fact correlated to the observation that these cells often rounded up.

To visualize morphological changes in the RacG-V12 transfected amoebae, the cells were analyzed by transmission electron microscopy. Cells harboring the Rac-V12 mutant displayed striking modifications of their surface including the spontaneous formation of vesicles (Fig. 7). These vesicles are similar to those formed by membrane budding which suggested that active membrane turnover was occurring without damage to the cell. All amoebae analyzed by TEM presented these budding structures. Their number varied between 3 and 19 per cell. The correlation between the disorganization of cytoskeleton and membrane budding in the RacG-V12 transfected amoebae raises the possibility that recruitment of F-actin under the membrane might, in some way, induce spontaneous membrane shedding or abortive uroids. This striking morphological change was never observed when amoebae were transfected with the NEO/CAT vector.

Capping, uroid induction and cytoskeleton rearrangement in RacG-V12 amoeba transfectants

To analyse the influence of *racG*-V12 expression on capping, transfected parasites were induced to cap surface receptors by

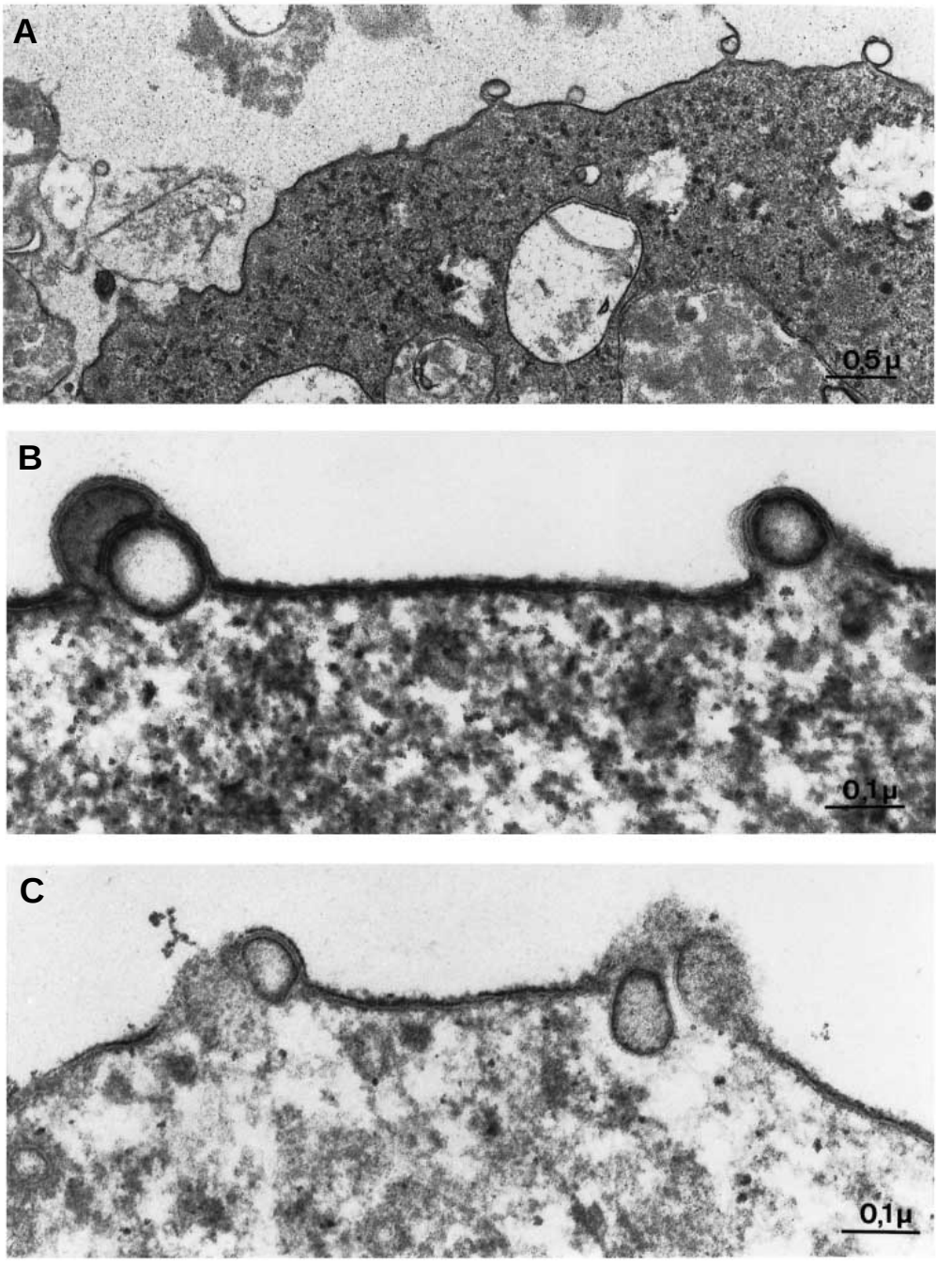

Fig. 7. Electron micrograph of a thin section of *E. histolytica* transfected with Eh*racG*-V12 mutant gene. Growing amoebae, transfected with the *racG*-V12 mutant allele, were fixed and treated for TEM. The amoebae displayed a regular plasma membrane with knob-like small vesicles apparently budding from the surface (A). Details of buds are shown in B and C. These images show buds that appear as very well-defined bodies emerging from the plasma membrane; they seem to extrude into the external medium. These buds were not observed in amoebae carrying the vector alone or wild-type amoebae.

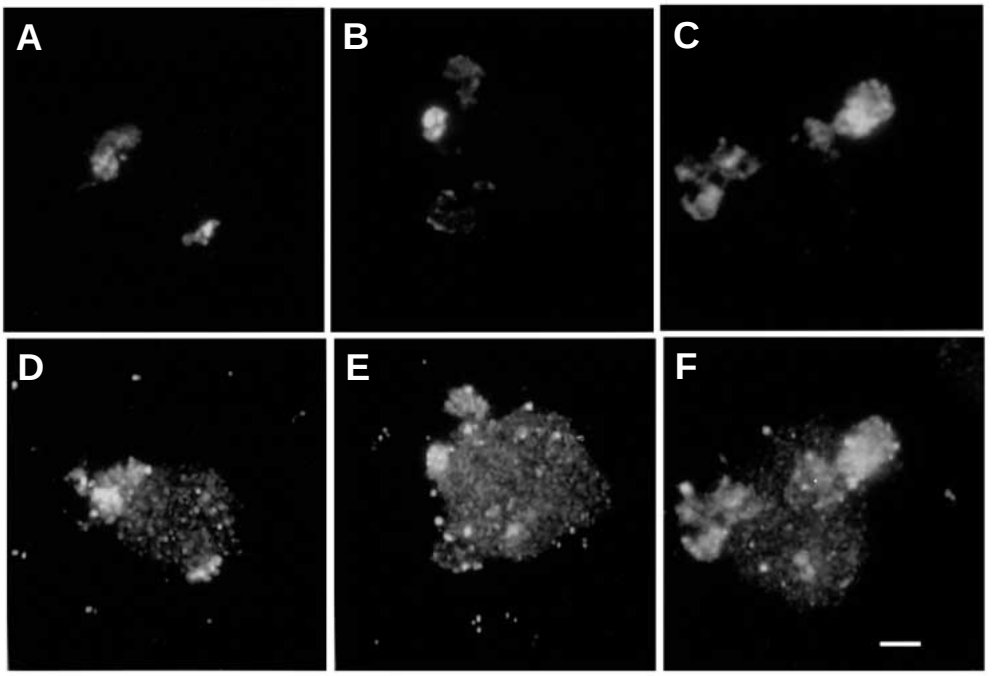

Fig. 8. Induction of capping in *E. histolytica* expressing the *racG*-V12 mutant allele. Capping was induced by incubation of the mutant amoebae with FITC-labeled Con A for 10 minutes at 37°C. Then cells were fixed and analyzed by CLSM as described in Materials and Methods. The micrograph shows the three-dimensional view of three RacG-V12 capping amoebae that present two uroids. (A,B,C) The uroids are detected by ConA fluorescence. (D.E.F) Labeling of RacG is shown. Both ConA and RacG colocalize in the uroids. Bar, 5 μM.

incubation with FITC-labeled ConA. Induction of capping was measured by the clustering of ConA-receptor complexes at the uroid region and localization of RacG was analyzed by CLSM. By observing fluorescent ConA (Fig. 8), we determined the frequency of amoebae presenting uroids in which ConA receptors were concentrated. Seventy percent of RacG-V12 transfectants showed ongoing capping; among these cells, 20% presented two or more uroids and 50% only one. Double staining of these capping amoebae showed that RacG colocalized with FITC-labelled ConA. In contrast, among amoebae transfected with the vector plasmid alone, 50% presented one uroid, and cells were never visualized with more than one appendage. After uroid induction amoebae were fixed and then stained for F-actin and for myosin II. Uroid formation during capping results, as expected, in the recruitment of the cytoskeleton to this appendage even in amoebae that presented more than one uroid (Fig 9). These results indicate that expression of the active form of RacG induced an uncontrolled formation of uroids, thus disturbing the polarization of *E. histolytica*. In addition, F-actin and myosin II are recruited to the uroid at the same time when amoebae were induced for capping of surface receptors by ConA, suggesting that these three proteins may undergo a common mechanism of regulation during capping.

DISCUSSION

E. histolytica is a lower eukaryotic cell known to destroy human tissues via cell contact killing. During infection, one of the parasitic defense mechanisms used to escape the host immune response is the capping of ligand-receptor complexes into the uroid region and eventual elimination of the complexes by membrane shedding. It has been demonstrated that the cytoskeleton is involved in this parasite adaptative response (Espinosa-Cantellano and Martinez-Palomo, 1994; Arhets et al., 1995). An important step towards understanding the mechanisms by which the cytoskeleton is regulated during capping, uroid formation, and membrane shedding is to identify and characterize the cytoskeleton-associated proteins that mediate these activities. Toward this goal, we have identified and characterized the small GTPase RacG which shares 85% similarity with human Rac1, a very well known regulator of the actin-rich cytoskeleton (Ridley, 1994). Our data suggest that the pathway through which the parasitic surface receptors communicate with the actomyosin cytoskeleton during capping involves EhRacG. It is likely that this protein works as part of a signal-transducing cascade. These data suggest that Rac plays a significant role in the capping process.

In amoebae activated for capping and uroid formation, we were able to localize RacG in the uroid region into which, in addition to the actomyosin complex, other cytoskeletal components are also recruited. This is the case for EhABP-120 (Vargas et al., 1996) which is necessary to cross-link actin. In addition, colocalization of the actomyosin complex and protein homologues of spectrin, α-actinin, plastin and ezrin has been observed in *E. histolytica* during capping (P. Arhets and N. Guillén, unpublished). It has been proposed that the ultimate targets for signal transduction molecules such as Rac are likely to be proteins that directly regulate actin polymerization or cross-link actin filaments, and, in turn, lead to membrane ruffling (Ridley, 1994). For instance, in addition to Rac, several other cytoskeletal proteins have been localized to membrane ruffles of mice fibroblasts. These proteins include ABP-280 (the human homologue of EhABP-120), α-actinin, plastin and ezrin (Ridley, 1994). It has been demonstrated that ezrin is phosphorylated on tyrosine residues in response to activation of tyrosine kinase receptors (Bretscher, 1989) and that plastin is phosphorylated at serine residues in response to interlukin-1 (Shiroo and Matsushima, 1990). In *E. histolytica*, EhRacG may regulate the activity of homologous cytoskeletal proteins

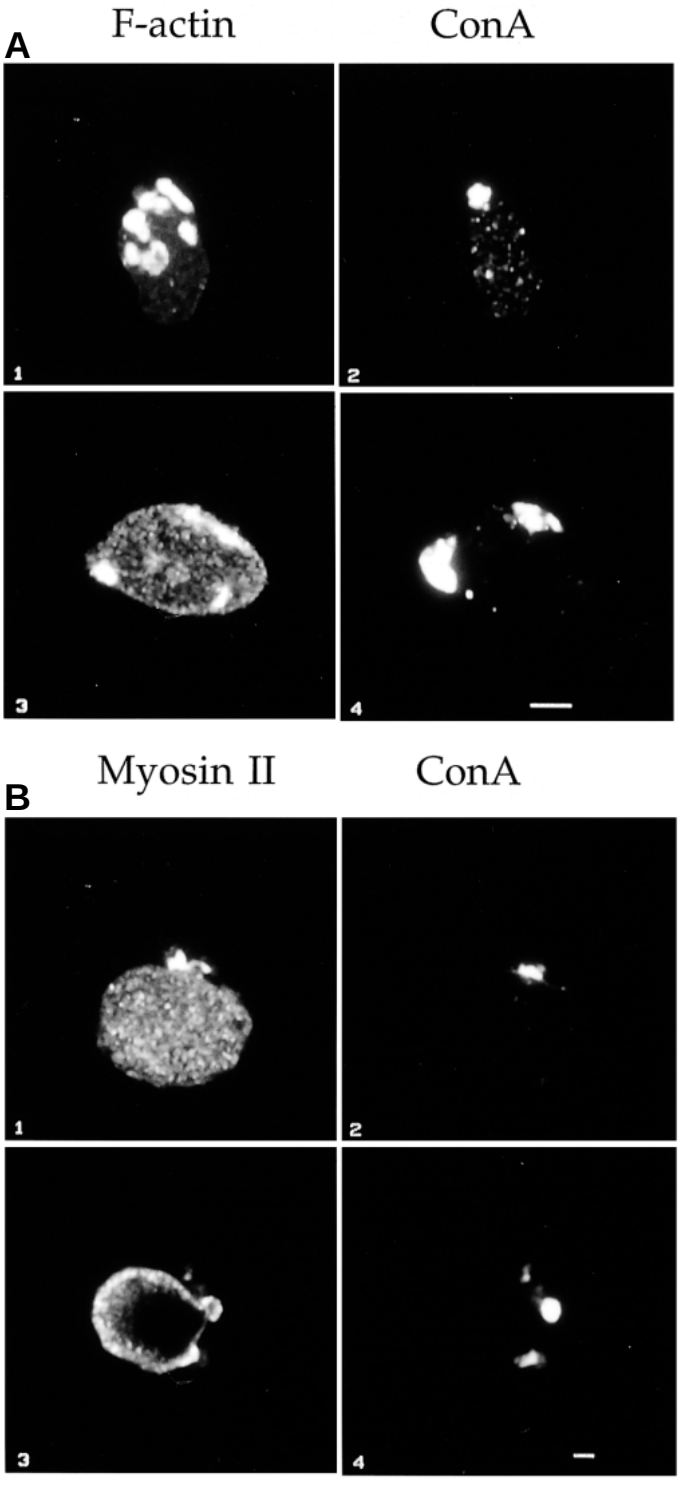

Fig. 9. Localization of F-actin and myosin II in *E. histolytica* expressing the *racG*-V12 mutant allele. Capping was induced by incubation of the mutant amoebae with FITC-labeled ConA for 10 minutes at 37°C. Then cells were fixed, stained for F-actin using phalloidin-rhodamine and for myosin II using anti-myosin II antibody, and then analyzed by CLSM as described in Materials and Methods. The micrograph shows the three-dimensional view of the wild-type (A1,2, B1,2) and a RacG-V12 (A3,4, B3,4) capping amoebae that present two uroids. In the right panels (A2,4, B2,4), the uroids are detected by the fluorescence of ConA. In the left panels, the labeling of F-actin (A1 and 3) or myosin II (B1 and 3) is represented. ConA, F-actin and myosin II are observed to colocalize in the uroids. Bars: 10 μM (A); 5 μM (B).

by phosphorylation events mediated by Rac-associated kinases (Burbelo et al., 1995). This hypothesis remains to be tested.

In *E. histolytica* inhibition of the activity of the actomyosin complex results in the simultaneous repression of both capping and formation of the uroid (Arhets et al., 1995). These findings indicate that there is a correlation between the mechanism responsible for capping and the mechanism necessary for folding and shedding of the membrane. In this work we observed that the expression of a constitutively active version of EhRacG leads to spontaneous membrane rearrangements and to bud formation. Amoebae display multiple small buds on the surface and, at the same time, F-actin is organized under the plasma membrane. These observations suggest a correlation between the enrichment of F-actin under the membrane and budding. Exacerbation of the membrane shedding phenomenon was observed when amoebae expressing the active form of RacG were induced to cap. Under such conditions, 20% of cells showed two or more uroids indicating major changes in the membrane and RacG, F-actin and myosin II were concentrated in the uroid. We cannot demonstrate at this point if membrane synthesis is activated under these conditions. Nevertheless, activity of extracellular factors which stimulate enzymes acting on membrane phospholipids has been correlated with actin polymerization and membrane ruffling (Ridley, 1994). Stimulation of the metabolism of phosphoinositides occurs in many signal transduction responses. It has been established that several actin-binding proteins bind phosphatidyl inositol (4,5) biphosphate (PIP2) and may transiently regulate actin polymerization (Ridley, 1994). A recent find has shown that the cytoskeleton purified from *E. histolytica* contains lipids kinases that lead to the formation of PiP2 in an in vitro assay. Although we have looked for differences between cytoskeletal fractions purified from ConA activated and unactivated amoebae, we did not observe differences in the quantity of PiP2 synthetized (N. Guillén, unpublished results), suggesting that there was no modification in the recruitment of PIP5 kinase in amoebae activated for capping. However, at this time, we do not know how these regulator components may induce turnover or membrane synthesis as well as activation of the cytoskeleton.

The finding that overexpression of active EhRacG can lead to both cytoskeletal reorganization and delocalization of uroids and can also affect the number of uroids formed, suggests that RacG is involved in the events responsabile for selection of uroid localization and for cell polarization, two markers of the morphogenic process of *E. histolytica*. A role in morphogenesis has also been attributed to Rac1 in *D. melanogaster*. Drac1 regulates the establishment of neuronal asymmetry and the normal F-actin distribution in embryonic flies (Luo et al., 1994). Interestingly, Drac1 appears as one of the most important homologues of EhRacG (Fig. 1).

Our results indicate that expression of an active form of EhRacG also affects parasite cytokinesis. This phenomenon may be explained by the delocalization of F-actin and loss of polarity in transfected amoebae. It is plausible that F-actin and/or other cytoskeletal proteins are not in the appropriate site for cellular

division. This phenotype is similar to that of RacE null mutants in *D. discoideum* (Larochelle et al., 1996). From the current data, these small GTPases are expected to participate in cytoskeletal rearrangements necessary to form a contractile ring.

In summary, we have described a novel phenotype of the small GTPase Rac and have proposed its role in the regulation of capping of surface receptors in a human parasite. Identification of EhRacG partners that play a role in the interaction of *E. histolytica* with human cells during movement and phagocytosis may provide new insights for cytoskeleton regulation during pathogenesis.

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