Regulation of the p21-activated Kinase-related *Dictyostelium* Myosin I Heavy Chain Kinase by Autophosphorylation, Acidic Phospholipids, and Ca\(^{2+}\)-Calmodulin*

(Received for publication, July 10, 1998, and in revised form, August 17, 1998)

Sheu-Fen Lee‡, Amjad Mahasneh, Marc de la Roche§, and Graham P. Côté¶

From the Department of Biochemistry, Queen’s University, Kingston, Ontario K7L 3N6, Canada

The *Dictyostelium* myosin I heavy chain kinase (MIHCK) is a member of the p21-activated kinase family (Lee, S.-F., Egelhoff, T. T., Mahasneh, A., and Côté, G. P. (1996) *J. Biol. Chem.* 271, 27044–27048). MIHCK incubated with MgATP in the absence of effectors incorporates 1 mol of phosphate/mol, resulting in an ~40-fold increase in kinase activity. Sequence analysis of tryptic peptides has identified the major site of phosphorylation as Ser-8. A peptide and a glutathione S-transferase fusion protein containing the Ser-8 phosphorylation site were good substrates for MIHCK, indicating that MIHCK can catalyze its own activation. Guanosine 5′-3′O-(thio)triphosphate (GTP\(\gamma\)S)-Rac1 stimulates MIHCK autophosphorylation and kinase activity 10-fold. Phosphatidyserine, phosphatidylinositol, and phosphatidylinositol 4,5-bisphosphate, but not phosphatidylcholine or sphingosine, were as effective as GTP\(\gamma\)S-Rac1 in enhancing MIHCK autophosphorylation and activity. Acidic lipids and GTP\(\gamma\)S-Rac1 induced the autophosphorylation of a similar set of sites as judged by two-dimensional tryptic peptide maps. It is proposed that GTP-Rac and acidic phospholipids function cooperatively to associate MIHCK with membranes. Ca\(^{2+}\)-calmodulin bound MIHCK and inhibited activation by acidic phospholipids but not by GTP\(\gamma\)S-Rac1. These studies reveal a number of similarities between the regulatory properties of the *Dictyostelium* and *Acanthamoeba* MIHCK, suggesting that the signaling pathways that control myosin I are conserved.

The small Rho family GTPases, Cdc42, Rac, and Rho, play a key role in controlling cell motility and morphology in many types of eukaryotic cells. In cultured fibroblasts, active GTP-Cdc42 and GTP-Rac promote the extension of actin filaments containing filopodia and lamellipodia, respectively, whereas in budding yeast, Cdc42 is involved in the control of cell polarity (reviewed in Refs. 1 and 2). At least some of the effects of Cdc42 and Rac are mediated through the direct activation of a family of serine/threonine protein kinases that includes the mamma-
Acanthamoeba MIHCK and identify the key autophosphorylation site responsible for the initial activation of Dictyostelium MIHCK as Ser-8.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP (grade I), GTP, S, TES, bovine serum albumin, bovine brain calmodulin, grade VI appraxe, aphisinoe (S-6859), and phosphoamino acid standards were obtained from Sigma. [γ-32P]ATP was from NEN Life Science Products, and phospholipids were from Serliday Research Laboratories (London, Ontario, Canada). Dictyostelium myosin ID and MIHCK were prepared as described (14, 23). Human Rac1 was expressed as a glutathione S-transferase (GST) fusion protein (kindly provided by Dr. Alan Hall, MRC Laboratory for Molecular Cell Biology, London, United Kingdom) and was purified over glutathione-Sepharose (Amersham Pharmacia Biotech). Rac1 was loaded with GTPγS as described (24).

**Phosphorylation Assays**—MIHCK activity was assayed in Buffer A (25 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol, 20 mM Tes, pH 7.0, 0.1 mg/ml bovine serum albumin) containing 0.25 mM [γ-32P]ATP (500 Ci/mmol). The concentrations of MIHCK, substrates, inhibitors, and activators for individual experiments are provided in the figure legends. Assays were initiated by the addition of MIHCK. To quantify the incorporation of 32P into proteins, aliquots of 10–20 μl were removed from the reaction, added to one-fifth volume of boiling SDS sample buffer (5% SDS, 30% sucrose, 2.5% β-mercaptoethanol), and subjected to SDS-PAGE (25). The appropriate protein band was then excised from the Coomassie Blue-stained gel and counted in scintillation fluid in a liquid scintillation counter. Incorporation of 32P into peptides was determined by spotting aliquots onto P-81 phosphocellulose paper (Whatman) (14). Conditions were chosen so that the incorporation of 32P into the substrate was linear over the time course of the experiment and proportional to the amount of MIHCK.

**Phosphopeptide Mapping and Identification of Tryptic Peptides from MIHCK**—MIHCK was incubated at a concentration of 30 μg/ml in Buffer A containing 0.1 mM [γ-32P]ATP (100 Ci/mmol) at 25 °C in the presence or absence of 0.5 mM phosphatidylserine (PS) and/or 0.2 mg/ml GTP-S-Rac1. After 1 h, the reactions were stopped by the addition of SDS sample buffer, and the MIHCK was electrophoresed on a 6% SDS-polyacrylamide gel and transferred electrophoretically to an Immobilon-P membrane (Millipore) (26). A narrow strip of Immobilon-P containing MIHCK (visualized with amido black) was excised, blocked for 20 min with 0.5% polyvinylpyrrolidine (BDH Chemicals) in 0.1 M acetic acid, washed extensively with water, cut into small pieces, and incubated with trypsin (2 μg) in 50 μl of 50 mM ammonium bicarbonate, pH 8, at 37 °C for 2 days, with the addition of a second aliquot of trypsin after 1 day (27). Following lyophilization, the sample was spotted onto a cellulose thin layer plate (Eastman Kodak) and subjected to electrophoresis in a Camag thin layer electrophoresis cell at pH 9 (acetic acid:formic acid:water, 8:2:90, v/v/v) for 1.5 h at 1000 V. Separation in the second dimension was by ascending chromatography in 1-butanol:pyridine:acetic acid:water (50:33:10:40) (27). Phosphoamino acid analysis was performed on MIHCK electrophoretically transferred to Immobilon-P (28). 32P-Labeled peptides and phosphopeptides were visualized by exposing the plates at −80 °C to Kodak X-AR film with an intensifying screen (NEN Life Science Products). To isolate sufficient material for sequence analysis, 200 μg of MIHCK was incubated in Buffer A containing 0.1 mM [γ-32P]ATP (600 Ci/mmol) at 25 °C for 1 h. The sample was subjected to SDS-PAGE, transferred to Immobilon-P, and digested as described above, except that the digestion volume was 1 ml, and a total of 20 μg of trypsin was added. After lyophilization, the digest was redissolved in 0.05% trifluoroacetic acid and chromatographed over a C5 reverse-phase high pressure liquid chromatography column (Amersham Pharmacia Biotech) and eluted with a gradient of increasing acetonitrile concentration. Sequence analysis of the 32P-labeled peptides was performed by the Biotechnology Service Center, Department of Clinical Biochemistry, University of Toronto.

**Binding Assays with Phospholipid Vesicles and Calmodulin**—Phospholipid vesicles were prepared essentially as described in (29). The vesicles were stored in 10% sucrose, 0.2 mM EDTA, 1 mM dithiothreitol, 10 mM Tes, pH 7.0, and phospholipid concentrations were determined by measurement of total phosphate (30). Binding assays were performed by mixing MIHCK with phospholipid vesicles (0.5 mM total lipid) in Buffer A at room temperature and centrifuging the mixture at 20,000 × g for 20 min in a Beckman TL100 centrifuge. The resulting pellets were washed once with Buffer A, resuspended to the same volume as the supernatants, and subjected, along with the supernatants, to SDS-PAGE. The calmodulin binding assay was performed by mixing 20 μl of bovine brain calmodulin-agarose beads (Sigma) with MIHCK in Buffer A containing 0.005% Tween 80 and either 25 μM CaCl2 or 300 μM EDTA. After a 30-min incubation at room temperature, samples were centrifuged for 2 min in an Eppendorf centrifuge at 2500 rpm. The resulting pellets were washed twice with Buffer A plus 0.005% Tween 80, resuspended to the original volume, and analyzed, with the supernatants, by SDS-PAGE. The relative amount of MIHCK in each fraction was quantified by scanning the Coomassie Blue-stained gel at 596 nm with an LKB 2202 Ultra laser densitometer.

**Miscellaneous Methods**—Extracts for immunoblotting were prepared by homogenizing D. discoideum AX-3 cells in 2 volumes of extraction buffer (14) containing either 20 or 100 mM KCl. Extracts were centrifuged at 200,000 × g for 30 min in a Beckman TL100 centrifuge to obtain supernatant and particulate fractions. Immunoblot analysis of proteins transferred to Immobilon-P with affinity purified anti-Dictyostelium MIHCK and anti-Dictyostelium myosin ID rabbit polyclonal antibodies were performed as described (16, 23). Protein concentrations were determined using the colorimetric assay of Bradford (31) with bovine serum albumin as the standard. Library screening and DNA manipulations were done according to standard procedures as described previously (16). To construct a fusion of GST with residues 1–42 of Dictyostelium MIHCK, the portion of the MIHCK cDNA coding for this fragment was amplified by polymerase chain reaction (PCR) from the full length MIHCK cDNA using the sense primer GAATTCATTGGTCAATCAAAAAGAG and the antisense primer TGGTACCATTGGTTTGAGCGGTATCATGAGGTCAATCATCTTGTGCC. The resulting 126-base pair product was cloned into pCR®2.1 (Invitrogen) and then cut with EcoRI and ligated into pGEX-4T-3 (Ams- terham Pharmacia Biotech). The recombinant fusion protein, designated GST-MIHCK–42, was expressed in Escherichia coli DH5α and purified over a glutathione-Sepharose affinity column (Amersham Pharmacia Biotech).

**RESULTS**

**Identification of the Phosphorylation Site Responsible for Activation of MIHCK**—Two-dimensional peptide maps of MIHCK phosphorylated to 1 mol/mol and digested with trypsin revealed the presence of a single major phosphopeptide (Fig. 1, None 1). Chromatography of the tryptic digest over a reverse-phase high pressure liquid chromatography column yielded two major peaks of radioactivity, both of which electrophoresed on thin layer plates with a mobility identical to the major phosphopeptide in Fig. 1 (data not shown). Amino acid sequence analysis of the first peak indicated the presence of a major peptide (QGNYDR) and a minor peptide (RVSMMR), whereas the second peak yielded RVSMMR as a unique sequence. The peptide QGNYDR lacks Ser and Thr residues and so must represent an unphosphorylated peptide that co-eluted with the phosphopeptide. It is not clear why the RVSMMR peptide was recovered in two distinct peaks from the reverse-phase column, although a possible explanation could be oxidation of one of the methionines to methionine sulfoxide (32).

The **Phosphorylation Site Is Located Close to the Amino Terminus of MIHCK and Is a Substrate for MIHCK**—A peptide sequence corresponding to RVSMMR is not present in the published MIHCK sequence (16). Isolation of additional cDNAs from a Dictyostelium cDNA library revealed that the amino-terminal 53 amino acids originally reported for Dictyostelium MIHCK is incorrect and may have arisen from sequencing artifact in which an irrelevant cDNA was ligated to a cDNA encoding the 5’-end of MIHCK. The corrected sequence replaces these 53 residues with the 9 residues MEGSQRVSM. The rest of the MIHCK sequence, starting at residue Met-10 (formerly Met-54) remains unchanged. The amino-terminal sequence has been confirmed by PCR analysis and has also been

27912 Regulation of Dictyostelium Myosin I Kinase
The panels show autoradiographs of two-dimensional tryptic peptide maps of MIHCK incubated with \[\text{[32P]}\text{ATP}\] in the absence of activators (None), with 0.2 mg/ml GTP-\gamma-S-Rac1 (Rac), with 0.5 mM phosphatidylyserine vesicles (PS), or with both GTP-\gamma-S-Rac1 and phosphatidylserine (PS + Rac). Details for the phosphorylation reaction, tryptic digestion, and peptide mapping are given under “Experimental Procedures.” The point at which the sample was applied is indicated as Origin, and the position of the chromatography buffer front is designated Front. The major phosphopeptide obtained in the absence of effectors is indicated as 1, and phosphopeptides observed only when phosphorylation was carried out in the presence of GTP-\gamma-S-Rac1 are indicated as 2 and 3.

Previously studies have suggested that the initial activation of MIHCK depends on an intermolecular autophosphorylation event (14). A fragment encompassing residues 1–42 of MIHCK, expressed as a GST fusion protein, was readily phosphorylated by MIHCK (Fig. 2A). Tryptic peptide maps of the phosphorylated GST-MIHCK1–42 fusion protein revealed the presence of a single phosphopeptide with a mobility identical to that of the RVSMMR phosphopeptide (data not shown). A synthetic peptide corresponding to residues 4–11 of MIHCK (but with Ser-4 replaced with Ala) was phosphorylated by MIHCK with a \(k_{\text{cat}}\) similar to that for a peptide based on the Dictyostelium myosin ID heavy chain phosphorylation site and a \(K_m\) about 6-fold higher (Table I). These results identify Ser-8 as a potential substrate for MIHCK.

### MIHCK Binds to Acidic Phospholipids and Membranes

The binding of MIHCK to vesicles composed of the electrically neutral phospholipid phosphatidylcholine (PC) or the negatively charged phospholipids PS or phosphatidylinositol (PI) was investigated using co-sedimentation experiments. MIHCK did not pellet in the absence of phospholipids or in the presence of charged phospholipids PS or phosphatidylinositol (PI) was investigated using co-sedimentation experiments. MIHCK did not pellet in the absence of phospholipids or in the presence of phosphatidylyserine (PS) or in the presence of both PS and PI (Fig. 2B).

### Acidic Phospholipids Stimulate MIHCK Autophosphorylation

Acidic phospholipids stimulate MIHCK autophosphorylation—PS vesicles dramatically enhanced the amount of phosphate incorporated into MIHCK, resulting in a lower electrophoretic mobility of the kinase on SDS-polyacrylamide gels (Fig. 3). The initial rate of autophosphorylation of MIHCK was
Regulation of Dictyostelium Myosin I Kinase

**TABLE I**

| Activator | Peptide Substrate | $k_{cat}$ | $K_m$ |
|-----------|-------------------|----------|-------|
| Rac1      | MIHCK 4–11        | 5        | 98    |
| PS        | MIHCK 4–11        | 5.4      | 116   |
| None      | myoD              | 0.3      | 16    |
| Rac1      | myoD              | 4.6      | 17    |
| PS        | myoD              | 4.7      | 19    |

**FIG. 3. Stimulation of MIHCK autophosphorylation by phosphatidylserine.** MIHCK (30 μg/ml) was incubated in Buffer A containing 0.25 mM $[^{32}P]ATP$ (500 Ci/mol) in the presence (closed circles) or absence (open circles) of PS (0.5 mM) as described under “Experimental Procedures.” Aliquots of 20 μl were removed at the indicated times and analyzed by SDS-PAGE as described under “Experimental Procedures.” The inset shows a Coomassie Blue-stained SDS gel of unphosphorylated MIHCK (0) and MIHCK incubated for 75 min in Buffer A with 0.25 mM ATP in the absence (−PS) and presence (+PS) of 0.5 mM phosphatidylserine. MIHCK phosphorylated in the presence of phosphatidylserine displayed a reduced electrophoretic mobility. Also significantly accelerated by PS (Fig. 3). Quantification of the linear portion of the MIHCK phosphorylation time course showed that PS enhanced the initial rate of MIHCK phospho-
ylation 35-fold, compared with a 20-fold acceleration by GTP$\gamma$S-Rac1 (Fig. 4A). The presence of 60 mM KCl completely inhibited the stimulatory effects of PS, indicating that the interaction of MIHCK with the acidic lipid is primarily electro-
static (Fig. 4A). Two other acidic phospholipids, PI and phosphatidylinositol 4,5-bisphosphate (PIP$_2$), also greatly en-
hanced the rate of MIHCK phosphorylation, whereas PC did not. The positively charged lipid sphingosine, which recently
has been shown to potently activate PKA autophosphorylation (33), had little effect on MIHCK (Fig. 4A). Vesicles had to
contain at least 70% PS to stimulate the rate of MIHCK phosphorylation and exceed 60% PS to bind MIHCK (Fig. 4B). PI
and PIP$_2$ had to comprise about 50% of total vesicle lipid to bind and activate MIHCK (data not shown).

**Acidic Phospholipids Stimulate MIHCK Activity and Autophosphorylation in a Manner Equivalent to Rac1—Stimulation of Dictyostelium MIHCK with a combination of PS and GTP$\gamma$S-Rac1 resulted in the same level of phosphate incorporation (9–10 mol of phosphate/mol) as was obtained with PS or GTP$\gamma$S-Rac1 separately. The lack of additive phosphorylation suggests that GTP$\gamma$S-Rac1 and PS promote the phosphoryla-
tion of a similar set of sites. Consistent with this view, tryptic digests of MIHCK autophosphorylated in the presence of PS, GTP$\gamma$S-Rac1, or a combination of PS and GTP$\gamma$S-Rac1 yielded qualitatively similar two-dimensional phosphopeptide maps (Fig. 1, 2, and 3) that were detected with GTP$\gamma$S-Rac1 activation but not with PS activation. These two peptides were also apparent when MIHCK was autophosphorylated in the presence of PS and GTP$\gamma$S-Rac1 (Fig. 1). Phosphorylation assays with peptide
substrates showed that PS and GTP\(\gamma\)S-Rac1 were independently able to fully stimulate the kinase activity of MIHCK (Table I). Stimulation of MIHCK with PS and GTP\(\gamma\)S-Rac1 together did not further enhance kinase activity.

The interaction between MIHCK and acidic phospholipids was influenced by the phosphorylation state of MIHCK. Unphosphorylated MIHCK bound to PS vesicles, but MIHCK phosphorylated to 10 mol of phosphate/mol remained in the supernatant fraction when centrifuged with PS vesicles (Fig. 5A). MIHCK containing 1 mol of phosphate/mol (prepared by phosphorylation in the absence of activators followed by addition of apyrase to remove ATP) sedimented with PS vesicles (Fig. 5A).

**MIHCK Interacts with Calmodulin in a Ca\(^{2+}\)-dependent Manner**—Unphosphorylated MIHCK sedimented with calmodulin-agarose beads in the presence, but not the absence, of Ca\(^{2+}\) (Fig. 5B). MIHCK containing 1 mol of phosphate/mol bound to the calmodulin-agarose in a Ca\(^{2+}\)-dependent manner, but MIHCK that fully autophosphorylated to 10 mol of phosphate/mol displayed only weak binding to Ca\(^{2+}\)-calmodulin (Fig. 5B). Ca\(^{2+}\)-calmodulin completely prevented the acidic phospholipid-stimulated activation of MIHCK (Fig. 5C). Both the rate of MIHCK autophosphorylation and the level of phosphate incorporation were returned to the levels obtained in the absence of acidic phospholipids. Ca\(^{2+}\)-calmodulin had no effect on the ability of MIHCK to incorporate 1 mol of phosphate/mol (i.e. phosphorylation of Ser-8). Furthermore, Ca\(^{2+}\)-calmodulin did not inhibit the GTP\(\gamma\)S-Rac1-stimulated activation of MIHCK (Fig. 5C).

**DISCUSSION**

The Dictyostelium MIHCK is a member of the PAK family and, like other members of this family, contains a conserved sequence motif that confers the ability to bind Cdc42 and Rac in a GTP-dependent manner (16). Previously, we have shown that the autophosphorylation and kinase activity of MIHCK can be significantly enhanced by GTP\(\gamma\)S-Cdc42 and GTP\(\gamma\)S-Rac1. In this paper, we identify two additional regulators of MIHCK, acidic phospholipids and Ca\(^{2+}\)-calmodulin, and provide further insight into the distinct two-stage process that converts MIHCK from an inactive to a fully active state.

During the first stage of activation, MIHCK is phosphorylated at a single site, now identified as Ser-8, resulting in a ~40-fold increase in kinase activity (Fig. 6). This step in the activation process occurs spontaneously in vitro when MIHCK is exposed to MgATP and is likely to be intermolecular in nature, because its rate is highly dependent on the MIHCK concentration (14). Ser-8 was found to be a good substrate for MIHCK both in the context of a small synthetic peptide (cor-
responding to residues 4–11 of MIHCK) and within a GST fusion protein containing the amino-terminal 42 residues of MIHCK. These results show that MIHCK has the ability to catalyze its own activation through an intermolecular autophosphorylation event, although they do not rule out the possibility that a separate MIHCK-activating kinase may exist. Ser-8 is located within a sequence (KRVSSMR) that resembles the activation site in the Dictyostelium myosin ID heavy chain (ARVSTY) as well as the consensus recognition sequences determined for γ-PAK (K/R)RXS (34) and the Acanthamoeba MIHCK (RXSXY) (18, 21). The tyrosine residue two amino acids carboxyl-terminal to the phosphorylated serine has been identified as an important recognition determinant for the Acanthamoeba MIHCK and, to a lesser extent, PAK. The replacement of this tyrosine with a methionine may account for the 6-fold higher $K_m$ of the MIHCK peptide relative to the myosin ID peptide (Table I). The intermolecular autophosphorylation of Ser-8 is clearly a key step in the activation of Dictyostelium MIHCK, so it is surprising that it proceeds in vitro without any requirement for regulatory molecules. It is possible that the rate of this reaction is controlled in vivo by a mechanism that dimerizes or otherwise concentrates MIHCK at a particular cellular location. In this respect it is interesting that Ste20p associates in vivo with Bmh1p and Bmh2p, the yeast homologs of the dimeric 14-3-3 proteins, and this interaction is required for Ste20p signaling during pseudohyphal development (35).

The finding that activation of Dictyostelium MIHCK results from the phosphorylation of a site close to the amino terminus is somewhat unexpected, because the activation of PAK (36, 37), Ste20p (38), and the isolated Acanthamoeba MIHCK catalytic domain (39) is closely tied to autophosphorylation of a Thr in a region of the catalytic domain termed the “activation segment” (reviewed in Ref. 40). The amino-terminal domains of PAK and the Acanthamoeba MIHCK do, however, have a pivotal role in suppressing kinase activity, because their removal by proteolysis significantly stimulates kinase activity (29, 41).

Recently, residues 83–149 of α-PAK have been identified as an autoinhibitory domain that potently inhibits GTP•S-Cdc42-mediated PAK activation (42). This region is highly conserved in Dictyostelium MIHCK (16), suggesting that the autoinhibitory function has been retained. It can be speculated that phosphorylation of Ser-8 perturbs the conformation of the amino-terminal domain such that the interactions between the autoinhibitory domain and kinase domain are weakened. Interestingly, phosphorylation of Ser-8 also promotes the binding of Dictyostelium MIHCK to a GTP•S-Rac1 affinity column (16). Thus, the conformational change that occurs upon Ser-8 phosphorylation not only leads to increased kinase activity but also primes MIHCK for the second stage of activation.

Partially active MIHCK is capable of phosphorylating exogenous protein and peptide substrates but cannot autophosphorylate beyond 1 mol of phosphate/mol. In this paper, we show that acidic phospholipids are as effective as GTP•S-Cdc42 in stimulating autophosphorylation to 10 mol of phosphate/mol and activating MIHCK activity. Both GTP•Rac1 and acidic phospholipids must alter the conformation of MIHCK so that serine and threonine residues that are hidden in the partially active MIHCK are exposed and available for autophosphorylation. The interaction of MIHCK with acidic phospholipids has the features of an electrostatic interaction, being dependent on the ionic strength and the percentage of acidic lipid in the membrane but independent of the chemical nature of the acidic lipid (43), indicating that the acidic lipid binding site on MIHCK must be composed of basic residues. The most highly positively charged section of MIHCK is located between residues 343–351 (KKKNKDKKK) and is immediately amino-terminal to the consensus Cdc42/Rac core binding motif (residues 355–368) (16). If residues 343–351 are partly responsible for the electrostatic interaction of MIHCK with acidic phospholipids, then its close proximity to the GTP-Cdc42/Rac binding site might provide an explanation for how these two chemically distinct activators could exert such similar effects on MIHCK. In contrast to MIHCK, PAK1 is potently activated by the positively charged lipid sphingosine and by some acidic lipids (phosphatidic acid and PI) but not by others (PS and PIP_2) (33), so that its interaction with phospholipids cannot be primarily electrostatic.

MIHCK can potentially associate with cellular membranes through its interaction with the small GTPase Rac, which is isoprenylated (44), and through electrostatic interaction with acidic phospholipids (Fig. 6). This arrangement is reminiscent of proteins such as MARCKS, Src, and Ki-Ras that require both hydrophobic and electrostatic interactions to form stable membrane associations (43). For these proteins, the hydrophobic interactions are contributed by a covalently attached lipid chain that can insert into the lipid bilayer and the electrostatic interactions by a cluster of basic amino acids that bind acidic lipids. By analogy to this model, we propose that a high affinity interaction of MIHCK with cellular membranes likely requires the presence of both acidic phospholipids and membrane-bound GTP•Rac (Fig. 6). Subcellular fractionation experiments demonstrated that the majority of MIHCK was not tightly bound to Dictyostelium membranes at physiological ionic strength (Fig. 2C) but during the process of membrane isolation active GTP•Rac may have been converted to inactive GDP•Rac. We would predict that the presence of an isoprenylated GTP•Rac should significantly enhance the binding of MIHCK to phospholipid vesicles and cellular membranes, and we are presently testing this hypothesis. Another prediction would be that interactions with membrane-bound GTP•Rac should dictate the cellular localization of MIHCK in vivo. Interestingly, a direct interaction with activated Cdc42 is required for Ste20p to properly localize to regions of polarized growth in yeast (45) but is not necessary for PAK to be recruited to focal complexes (42).

In this report we also show that the activation of Dictyostelium MIHCK by acidic phospholipids, but not by GTP•S-Rac, is prevented by Ca_{2+}•calmodulin. Competition between Ca_{2+}•calmodulin and acidic phospholipids for a common binding site has been described for Acanthamoeba MIHCK (22) and for the basic domain of MARCKS (46, 47). The affinity of MARCKS for both phospholipid vesicles and Ca_{2+}•calmodulin is decreased when serine residues in the basic domain are phosphorylated by PKC. By analogy to MARCKS, the basic domain of MIHCK responsible for the electrostatic interaction with acidic phospholipids (perhaps residues 343–352) may also be the Ca_{2+}•calmodulin binding site, and phosphorylation of sites within this region may be responsible for inhibiting the binding of MIHCK to both Ca_{2+}•calmodulin and acidic lipids. It will clearly be important to precisely map the Ca_{2+}•calmodulin binding and acidic phospholipid binding sites within Dictyostelium MIHCK. The present studies do, however, lead to the important conclusion that Ca_{2+}•calmodulin has a conserved function as an inhibitor of both the Dictyostelium and Acanthamoeba MIHCK. Indeed, Ca_{2+} may have a widespread and fundamental role in suppressing myosin I motile activity, because the vertebrate myosin I isoforms are inhibited at elevated levels of Ca_{2+} through a Ca_{2+}•dependent dissociation of the calmodulin light chains (48). Recently, the Acanthamoeba MIHCK has been reported to contain a putative Cdc42/Rac binding site (39), suggesting that its activity is likely to be regulated in some manner by these small GTPases. Thus, the framework for a conserved set of intracellular signals that are...
involved in the control of myosin I-driven motile events in lower eukaryotes is beginning to emerge. Further studies need to focus on the mechanism by which interactions with Ca²⁺-calmodulin, GTP-Cdc42/Rac, and acidic phospholipids are integrated to modulate the subcellular location and activity of these kinases.

REFERENCES
1. Hall, A. (1998) Science 279, 509–514
2. Leberer, E., Thomas, D. Y., and Whiteway, M. (1997) Curr. Opin. Genet. Dev. 7, 59–66
3. Lim, L., Manser, E., Leung, T., and Hall, C. (1996) Eur. J. Biochem. 242, 171–185
4. Sella, M. A., and Chernoff, J. (1997) Trends Cell Biol. 7, 162–167
5. Dharmawardhane, S., Sanders, L. C., Martin, S. S., Daniels, R. H., and Mooseker, M. S. (1995) J. Cell Biol. 133, 1265–1278
6. Moseker, M. S., and Chemay, R. E. (1995) Annu. Rev. Cell Dev. Biol. 11, 633–675
7. Pollard, T. D., Dobberstein, S. K., and Zet, H. G. (1991) Annu. Rev. Physiol. 53, 653–681
8. Novak, K. D., Peterson, M. D., Reed, M. C., and Titus, M. A. (1995) J. Cell Biol. 131, 1205–1221
9. Jung, G., Wu, X. F., and Hammer, J. A., III (1996) J. Cell Biol. 133, 365–383
10. Goodson, H. V., Anderson, B. L., Warrick, H. M., Pon, L. A., and Spudich, J. A. (1996) J. Cell Biol. 133, 1277–1291
11. Sement, W. M., and Mooseker, M. S. (1995) Cell Motil. Cytoskeleton 2, 87–92
12. Brzeska, H., and Korn, E. D. (1996) J. Biol. Chem. 271, 16983–16986
13. Wu, C., Lytwyn, V., Thomas, D. Y., and Leberer, E. (1997) J. Biol. Chem. 272, 30623–30626
14. Lee, S.-F., and Côte, G. P. (1995) J. Biol. Chem. 270, 11776–11782
15. Brzeska, H., Szczepanowska, J., Hsey, J., and Korn, E. D. (1996) J. Biol. Chem. 271, 27056–27062
16. Lee, S.-F., Egelhoff, T. T., Mahasneh, A., and Côte, G. P. (1996) J. Biol. Chem. 271, 27044–27048
17. Wu, C., Lee, S.-F., Furmaniak-Kazmierczak, E., Côte, G. P., Thomas, D. Y., and Leberer, E. (1996) J. Biol. Chem. 271, 31787–31790
18. Brzeska, H., Knaus, U. G., Wang, Z. Y., Bokoch, G. M., and Korn, E. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10022–10026
19. Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S., and Lim, L. (1994) Nature 371, 45–46
20. Brzeska, H., Lynch, T. J., and Korn, E. D. (1990) J. Biol. Chem. 265, 3591–3594
21. Brzeska, H., Lynch, T. J., Martin, B., Corigliano-Murphy, A., and Korn, E. D. (1990) J. Biol. Chem. 265, 16138–16144
22. Brzeska, H., Kulesza-Lipka, D., and Korn, E. D. (1992) J. Biol. Chem. 267, 23870–23875
23. Lee, S.-F., and Côte, G. P. (1993) J. Biol. Chem. 268, 20923–20929
24. Manser, E., Leung, T., and Lim, L. (1995) Methods Enzymol. 256, 130–139
25. Laemmli, U. K. (1970) Nature 227, 680–685
26. Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
27. Boyle, W. J., Van der Geer, P., and Hunter, T. (1991) Methods Enzymol. 201, 110–149
28. Kamps, M. P. (1991) Methods Enzymol. 201, 21–27
29. Brzeska, H., Martin, B., Kulesza-Lipka, D., Baines, I., and Korn, E. D. (1992) J. Biol. Chem. 267, 4949–4956
30. Ames, B. N. (1986) Methods Enzymol. 8, 115–118
31. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
32. Linde, S., Nielsen, J. H., Hansen, B., and Welinder, B. S. (1990) J. Chromatogr. 530, 29–37
33. Bokoch, G. M., Reilly, A. M., Daniels, R. H., King, C. C., Olivera, A., Spiegel, S., and Knaus, U. G. (1998) J. Biol. Chem. 273, 8137–8144
34. Turazon, P. T., Spanos, W. C., Gump, E. L., Monnig, C. A., and Traugh, J. A. (1997) Biochemistry 36, 16059–16064
35. Roberts, R. L., Misch, H. U., and Fink, G. R. (1997) Cell 89, 1055–1065
36. Benner, G. E., Dennis, P. B., and Mascaro, R. A. (1995) J. Biol. Chem. 270, 21121–21128
37. Manser, E., Huang, H. Y., Lou, T. H., Chen, X. Q., Dong, J. M., Leung, T., and Lim, L. (1997) Mol. Cell. Biol. 17, 1129–1143
38. Wu, C., Whiteway, M., Thomas, D. Y., and Leberer, E. (1995) J. Biol. Chem. 270, 15984–15992
39. Szczepanowska, J., Zhang, X., Herring, C. J., Qin, J., Korn, E. D., and Brzeska, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8503–8508
40. Johnson, L. N., Noble, M. E. M., and Owen, D. J. (1996) Cell 85, 149–158
41. Rudel, T., and Bokoch, G. M. (1997) Science 276, 1571–1574
42. Zhao, Z. S., Manser, E., Chen, X. Q., Chong, C., Leung, T., and Lim, L. (1998) Mol. Cell. Biol. 18, 2153–2163
43. McLaughlin, S., and Aderem, A. (1995) Trends Biochem. Sci. 20, 272–276
44. Kinsella, B. T., Erdman, R. A., and Maltese, W. A. (1991) J. Biol. Chem. 266, 9786–9794
45. Leberer, E., Wu, C. L., Leeuw, T., Fureest-Lieuvin, A., Segall, J. E., and Thomas, D. Y. (1997) EMBO J. 16, 83–97
46. Kim, J., Shishido, T., Jiang, X., Aderem, A., and McLaughlin, S. (1994) J. Biol. Chem. 269, 28214–28219
47. Swierczynski, S. L., and Blackshear, P. J. (1995) J. Biol. Chem. 270, 13436–13445
48. Coluccio, L. M. (1997) Am. J. Physiol. 273, C347–C359