Communication

Activation of Myosin-I by Members of the Ste20p Protein Kinase Family*

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The heavy chain of myosin-ID isolated from *Dictyostelium* was identified as an *in vitro* substrate for members of the Ste20p family of serine/threonine protein kinases which are thought to regulate conserved mitogen-activated protein kinase pathways. Yeast Ste20p and Cla4p and mammalian p21-activated protein kinase (PAK) phosphorylated the heavy chain to 0.5–0.6 mol of P_i/mol and stimulated the actin-dependent Mg^{2+}-ATPase activity to an extent equivalent to that of the Ste20p-like myosin-I heavy chain kinase isolated from *Dictyostelium*. PAK purified from rat brain required GTPγS-Cdc42 to express full activity, whereas recombinant mouse mPAK3 fused to glutathione *S*-transferase and purified from bacteria, and Ste20p and Cla4p purified from yeast extracts were fully active without GTPγS-Cdc42. These results suggest, together with the high degree of structural and functional conservation of Ste20p family members and myosin-I isoforms, that myosin-I activation by Ste20p family protein kinases may contribute to the regulation of morphogenetic processes in organisms ranging from yeast to mammalian cells.

The Ste20p and Cla4p protein kinases from *Saccharomyces cerevisiae* share an essential function for polarized growth during budding (1, 2). Ste20p plays an additional role in mediating the pheromone signal from the G-protein-coupled pheromone receptor to a downstream mitogen-activated protein (MAP)[1] kinase pathway (3). Both Ste20p and Cla4p belong to a growing family of homologous serine/threonine protein kinases believed to be involved in the regulation of conserved MAP kinase cas-

cades (for a review see Ref. 4). Several of these kinases share, in addition to the similar kinase domains, a binding site for members of the Rac and Cdc42 family of small G-proteins (5). These Rho-like small G-proteins are thought to play a role in mediating signals that trigger membrane ruffling and the assembly of focal adhesions in fibroblasts (6, 7). The mammalian homologs of Ste20p, the p21-activated kinases or PAKs (8–10), are therefore likely candidates for mediators of the effects of Rac and Cdc42 on changes in cellular morphology.

The recent identification of myosin-I heavy chain kinases (MIHCKs) in *Dictyostelium* and *Acanthamoeba* as novel members of the Ste20p family suggests that class I myosins might be important targets in Cdc42/Rac-mediated signaling pathways (11, 12). Myosin-I isoforms are unconventional single-headed, nonfilamenting myosins found in organisms ranging from protozoans such as *Acanthamoeba* and *Dictyostelium* to mammalian cells (for a review, see Ref. 13). Myosin-I molecules consist of a heavy chain and one or more light chains. The amino-terminal portion of the heavy chain forms a head domain displaying actin-stimulated Mg^{2+}-ATPase activity and generating the mechanochemical force for actin-based motile processes. The carboxyl-terminal portion forms a globular tail domain with binding sites for phospholipids and, in some isoforms, an ATP-independent filamentous actin binding site and a Src homology 3 (SH3) domain. The biochemical characterization of myosin-I isoforms from *Acanthamoeba* and *Dictyostelium* has demonstrated that phosphorylation of a single site in the heavy chain head domain is necessary to attain maximal actin-stimulated Mg^{2+}-ATPase activity and to contract and move actin filaments in *in vitro* assay systems (14–17).

In *S. cerevisiae*, Cdc42p is involved in two morphogenetic processes, budding during yeast proliferation and the formation of mating specific projections in response to pheromone (18, 19). Both events require polarized rearrangements of the actin cytoskeleton and transport of cell wall and membrane components to regions of active growth (20). Genetic experiments have indicated that the mechanochemical force for these processes might be generated by myosin-I isoforms whose heavy chains are encoded by the *MYO3* and *MYO5* genes (21). Thus, phosphorylation of these isoforms by Ste20p and Cla4p could act as an important mechanism in regulating morphogenetic processes in yeast.

Here, we show that the heavy chain of myosin-ID from *Dictyostelium* is phosphorylated and activated *in vitro* by the yeast Ste20p and Cla4p protein kinases and mammalian PAK. These results suggest, together with the high degree of structural and functional conservation of myosin-I and Ste20p protein kinase isoforms, that myosin-I regulation by Ste20p family protein kinases may be a general mechanism contributing to the control of morphogenetic processes in a variety of organisms ranging from yeast and protozoans to mammalian cells.

EXPERIMENTAL PROCEDURES

Yeast Strains and Manipulations—The *S. cerevisiae* strains used in this study were W303-1A (*MAT***a** *ade2 leu2 trp1 ura3 his3 can1*), YEL206 (W303-1A *ste20Δ-3::TRP1*), YEL276 (W303-1A *ste20Δ334–369*), and YEL252-1A (W303-1A *cla4Δ::TRP1*) (1, 22). Yeast manipulations were carried out as described (23).

Construction of Plasmids—To construct plasmid pDH129 carrying *CLA4* under control of the *GAL1* promoter, an *Xba*I to *Sac*II fragment of *CLA4* was amplified by the polymerase chain reaction (24) using the oligodeoxynucleotides ODH78 (5′-GC**TCTAGA**GCATGTCTCTTTCAG-

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[1] The abbreviations used are: MAP, mitogen-activated protein; GST, glutathione *S*-transferase; MIHCK, myosin-I heavy chain kinase; PAK, p21-activated protein kinase; mPAK3, mouse PAK3 isoform; PAGE, polyacrylamide gel electrophoresis; rPAK, rat brain PAK; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

CTGCAGCG-3′) and ODH80 (5′-TCC*CCGCGG*AATAGTTGTGTGCT-
TCATTCC-3′; the newly created *Xba*I and *Sac*II sites, respectively, are
underlined) as primers and plasmid pC2537 (2) as a template and
subcloned behind the *GAL1* promoter into pRS313GAL (25). The po-
lymerase chain reaction was used to change lysine residue 549 of Cla4p
to an arginine residue. First, two polymerase chain reactions were
performed using the oligodeoxynucleotide primer pairs ODH78 and
OCW32 (5′-GGTTGTTTAGATAAGACCATTTG*TCT*GATAGCAACTT-
TATCTC-3′) and OCW31 (5′-AGGAGATAAAGTTGCTATC*AGA*CAA-
ATGGTCTTATC-3′; the introduced nucleotide changes at positions
1646 and 1647 of *CLA4* are underlined) and ODH80, respectively, and
plasmid pDH129 as a template. The amplified fragments were then
purified, mixed, and used as templates for an additional polymerase
chain reaction using the oligodeoxynucleotides ODH78 and ODH80 as
primers. The resulting fragment was then digested with *Xba*I and *Sac*II
and cloned into pRS313GAL to yield plasmid pCW137.

A *GST-CLA4* fusion gene was constructed by cloning the *Bam*HI
fragment from nucleotide positions 418 to 1620 of *CLA4* (2) into plasmid
pGEX-KT (22). Plasmids pGST-mPAK3 carrying mPAK3 (10) fused to
GST in pGEX-KG and pDK-mPAK3^{K297R} carrying an mPAK3 mutant
with a change of lysine residue 297 to an arginine residue, were kindly
provided by S. Bagrodia, R. A. Cerione. The *mPAK3*^{K297R} cDNA was
fused to *GST* by subcloning a *Bam*HI fragment of pDK-mPAK3^{K297R}
into pGEX-4T3 (Pharmacia Biotech Inc.). Plasmids encoding GST fu-
sions of human and *S. cerevisiae* Cdc42 were kindly provided by A. Hall
and D. I. Johnson, respectively.

Preparation of Proteins and Antibodies—GST fusion proteins were
expressed in *E. coli* and purified over glutathione-Sepharose (22). The
GST-Cdc42 fusion proteins were loaded with GTPγS as described (26).
Ste20p and Cla4p were immunopurified from yeast cell extracts accord-
ing to the procedure previously described (22). Expression of Cla4p and
Cla4p^{K549R} from plasmids pDH129 and pCW137, respectively, was in-
duced by growing yeast transformants in selective media containing 4%
galactose. Rat brain PAK was purified as described (8). *Dictyostelium*
myosin-ID and MIHCK and rabbit skeletal muscle actin were purified
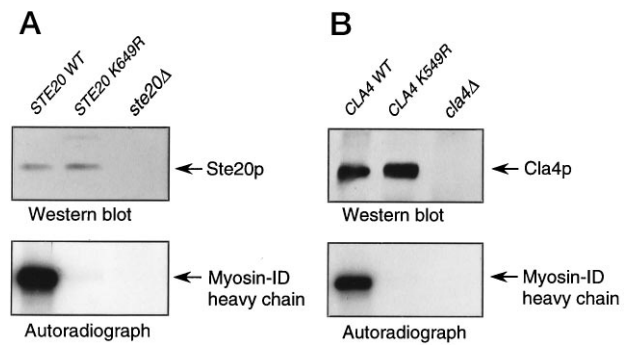as described (17, 27, 28).

Antibodies to Ste20p were as described previously (22). Antibodies to
Cla4p were raised by immunizing rabbits with GST fusion protein and
affinity-purified as described (22).

Myosin Phosphorylation and Mg^{2+}-ATPase Assays—Prior to the
phosphorylation reactions, the protein kinases were incubated in 20 mM
HEPES buffer, pH 7.5, containing 5 mM MgCl$_2$, 1 mM dithiothreitol, 5
μg/ml aprotinin, 5 μg/ml leupeptin, and 0.2 mM ATP (buffer A) for
20–40 min at 30 °C in order to stimulate autophosphorylation. In the
case for the protein kinases bound to beads, the beads were washed
twice with 20 mM HEPES buffer, pH 7.5, containing 5 mM MgCl$_2$, 1 mM
dithiothreitol, 5 μg/ml aprotinin, and 5 μg/ml leupeptin (buffer B). The
phosphorylation reactions were then carried out in 40 μl of buffer B
containing 0.2 mM [γ-^{32}P]ATP (1,000 Ci/mol) by addition of 0.3 μg of
myosin-ID. The reaction mixtures were incubated at 30 °C for the
indicated time periods, boiled for 5 min after addition of 40 μl of 2 ×
Laemmli buffer (29), and analyzed by SDS-PAGE (29) and autoradiog-
raphy. To quantify the phosphorylation reactions, the myosin-ID heavy
chain bands were excised from the dried gel and measured in a scintil-
lation counter. Chymotryptic phosphopeptide mapping of ^{32}P-labeled
myosin-ID was performed as described (30).

To measure stimulation of actin-dependent myosin Mg^{2+}-ATPase
activity, the autophosphorylated protein kinases were incubated with
0.7 μg of myosin-ID in 200 μl of buffer A for 20 min at 30 °C. Mg^{2+}-
ATPase assays were then performed for 20 min at 30 °C in 10 mM
HEPES buffer, pH 7.5, containing 5 mM MgCl$_2$, 1 mM dithiothreitol, and
1 mM [γ-^{32}P]ATP (1 Ci/mol) with and without 10 μM F-actin as described
(17).

RESULTS AND DISCUSSION

Recent studies showing that the myosin-I heavy chain ki-
nases (MIHCKs) from *Dictyostelium* and *Acanthamoeba* are
novel members of the Ste20p family of protein kinases (11, 12)
prompted us to test other members of this family for their
ability to phosphorylate and activate myosin-I. We found that
myosin-ID from *Dictyostelium* (27) is an *in vitro* substrate for
the yeast Ste20p and Cla4p protein kinases and mammalian
PAK. The yeast kinases immunopurified from yeast cell ex-
tracts phosphorylated the heavy chain of myosin-ID to about
0.6 mol of P$_i$/mol (Fig. 1). Similar levels of phosphate incorpo-
ration were found with mouse mPAK3, purified as a GST

A STE20 WT / STE20 K649R / ste20Δ ← Ste20p Western blot / ← Myosin-ID heavy chain Autoradiograph

B CLA4 WT / CLA4 K549R / cla4Δ ← Cla4p Western blot / ← Myosin-ID heavy chain Autoradiograph

Fig. 1. **Phosphorylation of myosin-ID heavy chain by Ste20p
and Cla4p.** Myosin-ID was phosphorylated for 20 min by Ste20p (*A*)
and Cla4p (*B*) immune complexes purified from yeast cell extracts as
described under "Experimental Procedures." The Ste20p and Cla4p
proteins were quantified by immunoblot analyses (*upper panels*). Myo-
sin-ID phosphorylation was analyzed by SDS-PAGE and autoradiogra-
phy (*lower panels*). To quantify phosphate incorporation, the labeled
bands were analyzed in a scintillation counter (0.6 mol of P$_i$/mol for both
Ste20p and Cla4p). The yeast strains were YEL206 transformed with
either plasmid pVTU-STE20 (3) expressing wild type Ste20p (*STE20
WT*), plasmid pVTU-STE20-K649R (22) expressing the catalytically
inactive Ste20p^{K649R} mutant (*STE20 K649R*) or the control vector
pVT102-U (*ste20Δ*), and YEL252-1A transformed with plasmids
pDH129 (*CLA4 WT*) and pCW137 (*CLA4 K549R*) expressing wild type
Cla4p and the catalytically inactive Cla4p^{K549R} mutant, respectively, or
the control vector pRS313GAL (*cla4Δ*).

fusion protein from *Escherichia coli* (Fig. 2), and *Dictyostelium*
MIHCK (17). Catalytically inactive mutant versions in which
the highly conserved lysine residues in the catalytic sites of the
kinases were replaced with arginine residues were unable to
phosphorylate the myosin-ID heavy chain (Fig. 1; data not
shown for mPAK3^{K297R}).

Mixtures containing the *Dictyostelium* kinase and either
Ste20p, Cla4p (data not shown), or GST-mPAK3 (Fig. 2), re-
spectively, produced similar phosphorylation levels. Moreover,
chymotryptic phosphopeptide maps of myosin-ID phosphoryl-
ated with either GST-mPAK3 or *Dictyostelium* MIHCK showed
identical phosphorylation patterns (data not shown). These
results suggest that the yeast and mouse kinases use the same
phosphorylation site as the *Dictyostelium* kinase.

Consistent with the view that the various members of the
Ste20p family all phosphorylate the same site on the myosin-I
heavy chain, Ste20p, Cla4p, and GST-mPAK3 were found to
stimulate the actin-dependent Mg^{2+}-ATPase activity of myo-
sin-ID to an extent equivalent to that of *Dictyostelium* MIHCK
(Fig. 3). No stimulation was found with the catalytically inac-
tive lysine to arginine mutant versions of these protein kinases
(Fig. 3). The magnitude of stimulation was identical after phos-
phorylation of myosin-ID with either GST-mPAK3 alone or a
mixture of GST-mPAK3 and *Dictyostelium* MIHCK (Fig. 3)
providing further support for the notion that the different
members of the Ste20p family phosphorylate the same regula-
tory site. An equivalent stimulation was observed with GTPγS-
Cdc42 activated rPAK purified from rat brain (Fig. 3).

Ste20p, Cla4p, and GST-mPAK3 could fully stimulate myo-
sin-ID actin-dependent Mg^{2+}-ATPase activity in the absence of
GTPγS-loaded Cdc42, and the previously characterized
Ste20p$^{Δ334–369}$ mutant in which Cdc42p binding to Ste20p was
prevented by removal of its Cdc42p binding site (1) was fully
active (Fig. 3). In contrast, rPAK purified from rat brain re-
quired GTPγS-Cdc42 to express full activity (Fig. 3). These
results are consistent with the finding that mutational preven-
tion of Cdc42p binding to Ste20p does not affect its *in vitro*
kinase activity (1, 31) and might distinguish the yeast kinases
from the rat brain homolog which has been shown to be stim-
ulated by activated Rac and Cdc42 *in vitro* (8). However, in
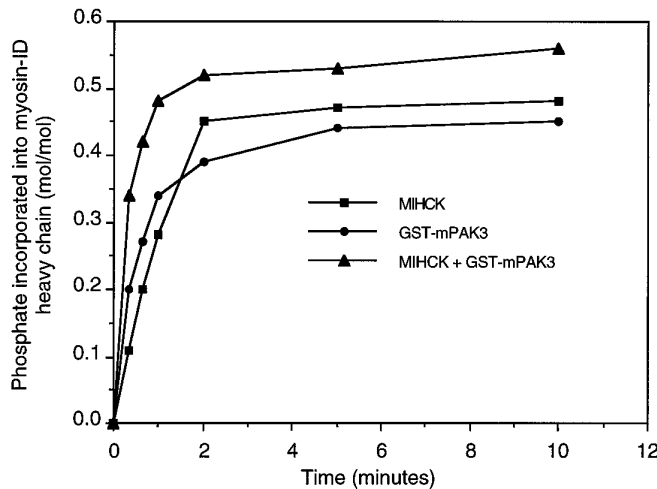
FIG. 2. **Phosphorylation of myosin-ID heavy chain by GST-mPAK3.** Myosin-ID was phosphorylated by either GST-mPAK3 (●), *Dictyostelium* MIHCK (■), or a mixture of both (▲). Incorporation of phosphate into the myosin-ID heavy chain was then quantified as described under "Experimental Procedures."
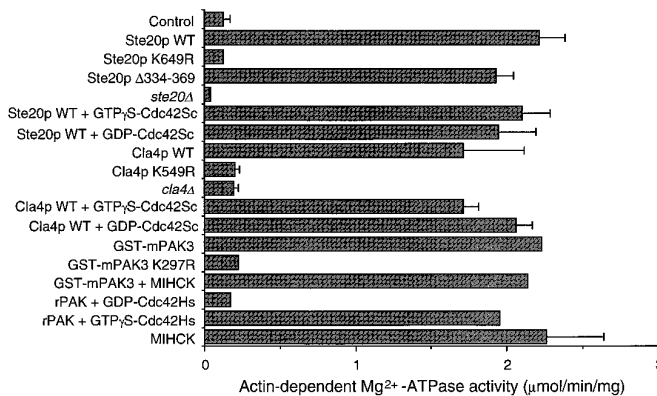

FIG. 3. **Stimulation of the actin-dependent Mg^{2+}-ATPase activity of myosin-ID by Ste20p family protein kinases.** Actin-dependent Mg^{2+}-ATPase activities were determined as the difference between the Mg^{2+}-ATPase activity in the presence and absence of actin. The assays were performed either without protein kinases (control) or with the indicated protein kinases, either as immune complexes isolated from yeast cell extracts, GST fusions isolated from *E. coli*, or purified proteins from rat brain (rPAK) or *Dictyostelium* (MIHCK). Ste20p and Cla4p immune complexes were isolated from wild type and mutant yeast strains as described in Fig. 1. The Ste20p$^{\Delta334-369}$ mutant version deleted for the Cdc42p binding domain was isolated by immune precipitation from strain YEL276. Where indicated, the reaction mixtures contained GTPγS or GDP-loaded yeast (*Sc*) or human (*Hs*) Cdc42 fused to GST and isolated from *E. coli*. The kinase preparations alone were free of Mg^{2+}-ATPase activity, and the kinases did not stimulate the basal myosin-ID Mg^{2+}-ATPase activity in the absence of actin (data not shown). The data represent mean values ± S.D. of 3–8 experiments or mean values of 2 experiments.

view of the finding that Cdc42p binding to Ste20p is needed for proper intracellular localization of Ste20p in yeast cells in regions of active growth (1, 31), it is conceivable that Cdc42p might play an important role for activation of myosin-I in yeast *in vivo*.

The phosphorylated residues responsible for activation of *Acanthamoeba* myosin-I isoforms have been identified as either serine or threonine residues within the sequence motif (K/R)X(S/T)XY that is conserved in the heavy chains of all myosin-I isoforms from *Acanthamoeba, Dictyostelium*, and *Aspergillus*, and in the *Drosophila* and mammalian class VI myosins (32, 33). It seems reasonable to suppose that the corresponding serine residue in the heavy chain of *Dictyostelium* myosin-ID is the regulatory residue phosphorylated by the members of the Ste20p family of protein kinases.

This regulatory residue is also conserved in the functionally redundant *S. cerevisiae* myosin-I isoforms encoded by the *MYO3* and *MYO5* genes (21, 34, 35). We have observed that the *in vivo* function of Myo3p is lost after changing the conserved serine residue 357 to an alanine residue, but is unaltered when the same residue is changed to an aspartate residue, suggesting that Myo3p needs a negative charge at this position for normal function.[2] It therefore appears likely that Myo3p and Myo5p are physiological targets of the Ste20p and Cla4p protein kinases in yeast. This is an attractive assumption in view of the requirement of Ste20p and Cla4p for polarized growth during cytokinesis (2), the role of Myo3p and Myo5p in polarized rearrangement of the actin cytoskeleton during budding (21), and the localization of both Ste20p and Myo5p to actin cortical patches (1, 21).

In all currently known mammalian myosin-I isoforms, the phosphorylated residue of *Acanthamoeba* myosin-I corresponds to aspartate or glutamate residues (33). The most likely candidates for targets of mammalian PAKs are therefore the mammalian class VI isoforms in which the potential regulatory phosphorylation site is conserved (33). Regulation of more than one class of myosins by Ste20p family protein kinases is suggested by the findings that PAK and the *Acanthamoeba* MIHCK can activate smooth muscle myosin-II by phosphorylating the light chain (36, 37).

The high degree of evolutionary sequence conservation of myosin-I and Ste20p isoforms suggests that the here described myosin-I regulation by members of the Ste20p protein kinase family may be a general mechanism found in organisms extending from yeast and protozoans to higher eukaryotes. Genetic experiments in *S. cerevisiae, Schizosaccharomyces pombe*, and *Drosophila* have demonstrated that members of the Ste20p family are involved in morphogenetic processes that control cell polarity (2, 38–40). As mediators of Rac and Cdc42, the PAK isoforms are expected to play a similar role in mammalian cells. In view of the well known function of yeast Ste20p in linking external signals to MAP kinase cascades that regulate transcriptional activation in two developmental pathways, conjugation and morphological switching induced by signaling through either the βγ subunits of the mating-response G-protein or Cdc42p, respectively (3, 31, 41–44), our results raise the intriguing possibility that Ste20p family protein kinases may provide an efficient mode of coordinating transcriptional ac-
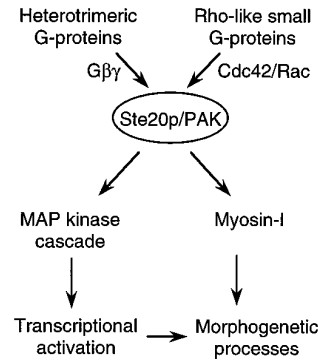

FIG. 4. **Proposed model for the dual role of Ste20p family protein kinases in stimulation of MAP kinase cascades and myosin-I.** We propose that Ste20p family protein kinases may play a role in coordinating transcriptional activation of genes by MAP kinase cascades with morphogenetic changes driven by myosin-I in response to signals mediated by either the βγ subunits of heterotrimeric G-proteins or by Rho-like small G-proteins of the Cdc42/Rac subfamily.

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[2] C. Wu, V. Lytvyn, D. Y. Thomas, and E. Leberer, unpublished observations.

tivation of genes by MAP kinase cascades with morphological processes driven by myosin-I in response to signals mediated by heterotrimeric and Rho-like small G-proteins (Fig. 4).

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