NOTE

WD Repeat Domain of *Dictyostelium* Myosin Heavy Chain Kinase C Functions in both Substrate Targeting and Cellular Localization

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Myosin II disassembly in *Dictyostelium discoideum* is regulated by three structurally related myosin heavy chain kinases (myosin II heavy chain kinase A [MHCK-A], -B, and -C). We show that the WD repeat domain of MHCK-C is unique in that it mediates both substrate targeting and subcellular localization, revealing a target for regulation that is distinct from those of the other MHCKs.

The ability of a cell to undergo highly specific modifications in shape during processes such as cytokinesis, cell migration, cell adhesion, and receptor capping is dependent, in large part, on the proper control of where and when myosin II contracts actin filaments in the cell (3, 4). In *Dictyostelium discoideum*, myosin II filament disassembly is regulated by at least three myosin II heavy chain kinases (myosin II heavy chain kinase A [MHCK-A], MHCK-B, and MHCK-C). The *Dictyostelium* MHCKs possess alpha kinase domains and carboxyl-terminal WD repeat domains (11, 13, 17). The WD repeat domains of MHCK-A and MHCK-B facilitate myosin II heavy chain phosphorylation by these kinases by binding directly to myosin II filaments (14, 15). However, the WD repeat domains play no detectable role in determining the subcellular localization of these kinases. Similar functions for the WD repeat domain of MHCK-C have not been explored, and there is nothing known about the signaling events regulating MHCK-C localization and activity, thus limiting comparisons among the MHCKs that could ultimately reveal distinct functions and mechanisms of regulation for these seemingly redundant enzymes.

**MHCK-C requires its WD repeat domain to phosphorylate MHC.** We explored the hypothesis that the predicted structural similarity of the WD repeat domain of MHCK-C with those of MHCK-A and -B reflects a shared function, such as substrate targeting (7). To this end, we examined the ability of FLAG-tagged full-length MHCK-C or a truncation lacking the substrate targeting (7). To this end, we examined the ability of FLAG-tagged full-length MHCK-C or a truncation lacking the WD repeat domain (MHCK-C-Δ-WD) to phosphorylate MHC substrate (0.42 μM) as well as to phosphorylate a peptide substrate (MH-1; 50 μM) that is phosphorylated by other alpha kinases in a WD repeat domain-independent manner (Fig. 1A and B) (15). Full-length MHCK-C phosphorylated MHC in a time-dependent manner and with a stoichiometry of approximately 2 moles of phosphate transferred per mole of MHC (Fig. 1C). In comparison, the MHCK-C-Δ-WD truncation was unable to phosphorylate MHC to a significant level, even after 20 min of incubation (Fig. 1C). In contrast, both MHCK-C and MHCK-C-Δ-WD phosphorylated the MH-1 peptide robustly and to essentially the same level (Fig. 1D), demonstrating that the catalytic activity of the MHCK-Δ-WD truncation is intact even in the absence of the WD repeat domain, and by extension, that the WD repeat domain is required for MHCK-C phosphorylation of MHC.

The WD repeat domain is required for MHCK-C functionality in cells. To examine further the role of the WD repeat domain in defining the activity of MHCK-C, *Dictyostelium* cell lines overexpressing FLAG-tagged full-length MHCK-C (MHCK-C++ cells) or truncations either lacking the WD repeat domain (MHCK-C-Δ-WD++ cells) or containing only the WD repeat domain (MHCK-C-WD++ cells) were monitored for growth in suspension culture. These cell lines exhibited expression levels at least 14-fold-higher than that of endogenous MHCK-C (see Fig. S1A and S1B in the supplemental material). We found that MHCK-C++ cells exhibited dramatically reduced rates of proliferation in suspension culture (Fig. 2A), accompanied by increased cell multinuclearity (Fig. 2B and C) and loss of cytoskeleton-associated myosin II (see Fig. S1C in the supplemental material). In contrast, the MHCK-C-Δ-WD++ and MHCK-C-WD++ cells grew at essentially the same rate as AX2 and MHCK-C-null cells, with very few multinucleated cells (Fig. 2). We also found that MHCK-C-Δ-WD++ cells, unlike MHCK-C++ cells and MHCK-null cells (6), completed the developmental cycle and formed fruiting bodies containing viable spores (data not shown).

**MHCK-C translocates to the cell cortex upon cAMP stimulation.** To gain insight into the signaling pathways involved in regulating MHCK-C activity and localization, we examined the effect of cyclic AMP (cAMP) stimulation on the localization of green fluorescent protein (GFP)-tagged MHCK-C (GFP-MHCK-C). It is noteworthy that the expression of GFP-MHCK-C for these experiments (see Fig. S2A in the supplemental material) did not affect the levels of cytoskeleton-associated proteins or the overall morphology of the AX2 cell line. A cAMP pulse, which is known to induce cytokinesis, was sufficient to drive MHCK-C from the cytosol to the cell cortex (Fig. 3). This cAMP-driven translocation of MHCK-C was not observed in the absence of the WD repeat domain, as reflected by the localization of the MHCK-C-Δ-WD truncation (Fig. 3). These results indicate that the WD repeat domain of MHCK-C is required for the cellular localization and activity of this myosin heavy chain kinase.

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FIG. 1. Analysis of MHCK-C and MHCK-C-Δ-WD activities toward myosin II substrate and MH-1 peptide substrate. (A) Domain organization of MHCK-C. Amino acid numbering refers to the numbering in the GenBank protein entry (GenPept accession number AAC31918). The SNPQ-rich region is the indicated region that possesses a high proportion of serines, asparagines, prolines, and glutamines. Detailed schematic representations of all of the Dictyostelium MHCKs can be viewed in reference 1. The regions of the kinase contained in the MHCK-C-Δ-WD and MHCK-C-WD truncations are indicated by brackets. (B) Coomassie blue-stained SDS-polyacrylamide gels of 2 μg of purified FLAG-tagged MHCK-C (left lane) and the MHCK-C-Δ-WD (right lane). FLAG-tagged full-length MHCK-C (FLAG-MHCK-C) and the MHCK-C-Δ-WD truncation were purified to homogeneity from the described Dictyostelium cell lines via the affinity chromatography protocol described by Liang et al. (9). MHCK-C-null cells expressing FLAG-MHCK-C were obtained from Tom Egelhoff (Cleveland Clinic Foundation) (9). Generation of plasmid and cell lines for the expression of FLAG-tagged MHCK-C-Δ-WD followed the strategy detailed by us previously (12). (C) The stoichiometry of Dictyostelium myosin II (0.42 μM) phosphorylation by MHCK-C (●) and MHCK-C-Δ-WD (■) was assayed over time as described previously (15). (D) The MHCK-C (●) and MHCK-C-Δ-WD (■) proteins were assessed for MH-1 peptide phosphorylation over time using the filter binding method (15). The values plotted in the graphs in panels C and D represent the mean values ± standard errors of the means (error bars) determined from three independent experiments. MolesPi, moles of inorganic phosphate.
myosin II (see Fig. S2C in the supplemental material) and did not induce the myosin II-null phenotype observed with MHCK-C$^{++}$ cells (see Fig. S2B in the supplemental material). For localization experiments, these cells were prepared and then stimulated with cAMP (50 μM) as described by us previously (16). In unstimulated cells, GFP-MHCK-C exhibits a relatively diffuse distribution (Fig. 3A, 0-s panel). About 40 seconds after stimulation, GFP-MHCK-C becomes detectably enriched throughout the cell cortex, with peak enrichment observed about 60 s after stimulation (Fig. 3A, white arrows). Chemoattractant-induced translocation was undetectable in cells lacking myosin II or treated with latrunculin A (5 μM, 20 min) (see Fig. S3 in the supplemental material). Complementary Triton X-100 cytoskeleton fractionation assays (performed exactly as described previously [16]) revealed that cAMP stimulation of Dictyostelium AX2 cells expressing only endogenous MHCK-C leads to a greater than 3-fold increase in the amount of kinase associated with the cytoskeleton-enriched fraction, with a timing that lags slightly behind that of myosin II (Fig. 3B and C). Taken together, these data are the first to demonstrate that the activation of chemoattractant signaling pathways can induce MHCK-C recruitment to the cell cortex.

The cellular localization of MHCK-C is mediated by its WD repeat domain. We next examined the structural determinants required for proper localization of MHCK-C in the cell. We found that GFP-C-Δ WD, which lacks the WD repeat domain, is distributed diffusely throughout the cell, with no cortical enrichment observed about 60 s after stimulation (Fig. 3A, white arrows). Chemoattractant-induced translocation was undetectable in cells lacking myosin II or treated with latrunculin A (5 μM, 20 min) (see Fig. S3 in the supplemental material). Complementary Triton X-100 cytoskeleton fractionation assays (performed exactly as described previously [16]) revealed that cAMP stimulation of Dictyostelium AX2 cells...
FIG. 3. MHCK-C is recruited to the cell cortex in response to cAMP stimulation. (A) Localization of GFP-tagged MHCK-C was monitored before (0 s) and after the addition of a uniform and saturating concentration of cAMP (50 μM) to the cells. Images were collected every 7 seconds, up to 140 seconds, using an Olympus IX70 microscope system and a UPlan 40× oil objective lens. White arrows indicate cells exhibiting particularly robust translocation responses. MHCK-C null cells expressing GFP-MHCK-C were obtained from Tom Egelhoff (Cleveland Clinic Foundation) (9). Bar, 20 μm. (B) Chemotactant-responsive AX2 (wild-type) cells were stimulated with cAMP (50 μM), and cytoskeleton-enriched pellets were collected at the indicated number of seconds following stimulation. Pellet fractions were subjected to SDS-PAGE, and the relative levels of actin and myosin II in these fractions were assessed by densitometric analysis of Coomassie blue-stained gels. Recruitment of endogenous MHCK-C to the cytoskeletal fraction was determined by Western blotting and densitometric quantification of band intensity. The Western blot lane corresponding to the 120-s time point is from the same experiment but was analyzed with a separate blot. The values plotted represent the means ± standard errors of means (error bars) from 7 separate experiments. (C) Representative Coomassie blue-stained gel and Western blot from a Triton X-100-resistant cytoskeleton fraction assay. The positions of myosin II (243 kDa) and actin (43 kDa) are indicated by arrows to the right of the gel, and the Western blot above the gel was probed with anti-MHCK-C antibody (9).
chemotaxis or random migration (Fig. 4B), thus demonstrating that the WD repeat domain contains all of the determinants required for normal localization of MHCK-C in the cell. As with the full-length kinase, no detectable translocation of GFP-C-WD was observed in cells lacking MHC or cells that were treated with latrunculin A (see Fig. S3 in the supplemental material).

Collectively, our results are the first to show that a WD repeat domain can function both in directing kinase phosphorylation of a substrate and in mediating subcellular localization and thus reveal an important distinction from the functions of the same domain of the MHCK-A isoform. In the case of MHCK-A, the WD repeat domain shares substrate targeting, but not localization, activity with that of MHCK-C (14, 15). Instead, MHCK-A localization to the actin-rich anterior of the cell is mediated by an extended region at the amino terminus of the kinase that is predicted to form mainly coiled-coil structures (12). The findings reported here, along with those showing MHCK-C recruitment to the cleavage furrow (11), support a model in which the assembly of myosin II into the cell cortex is the chief determinant for the MHCK-C translocation to the rear of cells undergoing chemotaxis or to the cleavage furrow during cytokinesis (11). At this time, there are a number of candidate effectors of the MHCKs in Dictyostelium; however, none have been shown to alter kinase activity or localization via a direct interaction or by phosphoryla-

**FIG. 4.** Analysis of MHCK-C truncations for cAMP-induced cortical translocation. (A) GFP-MHCK-C (full-length), GFP-C-Δ-WD, and GFP-C-WD were analyzed for chemoattractant-induced changes in cellular localization. Images were collected before the addition of cAMP (0 s) and at 60 s after the addition of a uniform and saturating concentration of cAMP (50 μM) to the cells. Cell lines expressing GFP-tagged MHCK-C-Δ-WD or GFP-C-WD were generated in the same manner as described for cells expressing FLAG-tagged truncations, except that the pTX-GFP vector was used in place of pTX-FLAG (8). (B) Serial images of GFP-C-WD localization in Dictyostelium cells undergoing chemotaxis or random migration. The large white arrow shows the direction of chemotaxis toward an aggregation center. Small white arrows point to sites of GFP-MHCK-C enrichment. Bars, 20 μm.
tion (or other modification) (2, 5, 10). Our results suggest that such signals could influence both MHCK-A and MHCK-C, but with very different responses from the kinases due to their very different localization domains.

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