A Specific Interaction between Muskelin and the Cyclin-dependent Kinase 5 Activator p39 Promotes Peripheral Localization of Muskelin*

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Previous studies implicate cyclin-dependent kinase 5 in cell adhesion and migration of epithelial cells of the cornea and lens. To explore molecular interactions underlying these functions, we performed yeast two-hybrid screening of an embryonic rat lens library for proteins that interact with cyclin-dependent kinase 5 and its regulators, p35 and p39. This screen identified a specific interaction between p39 and muskelin, an intracellular protein known to affect cytoskeletal organization in adherent cells. Immunohistochemistry detected muskelin in the developing lens and in other tissues, including brain and muscle. Glutathione S-transferase pull-down experiments and co-immunoprecipitations confirmed the specificity of the p39-muskelin interaction. Deletion analysis of p39 showed that muskelin binds to the p39 C terminus, which contains a short insertion (amino acids 329–366) absent from p35. Similar analysis of muskelin mapped the interaction with p39 to the fifth kelch repeat. Co-expression of p39 and muskelin in COS1 cells or lens epithelial cells altered the intracellular localization of muskelin, recruiting it to the cell periphery. These findings demonstrate a novel interaction between muskelin and the cyclin-dependent kinase 5 activator p39 and suggest that p39 may regulate the subcellular localization of muskelin.

Cyclin-dependent kinase 5 (Cdk5) is a unique member of the cyclin-dependent kinase family. Unlike other cyclin-dependent kinases, its cellular functions are not related to the regulation of cell cycle progression (1), and its activation requires a regulatory protein (either p35 or p39) that is not a member of the cyclin family. Cdk5 expression is widespread, but its kinase activity is found predominantly in the central nervous system and its regulators, p35 and p39. This screen identified a specific interaction between p39 and muskelin, an intracellular protein known to affect cytoskeletal organization in adherent cells. Immunohistochemistry detected muskelin in the developing lens and in other tissues, including brain and muscle. Glutathione S-transferase pull-down experiments and co-immunoprecipitations confirmed the specificity of the p39-muskelin interaction. Deletion analysis of p39 showed that muskelin binds to the p39 C terminus, which contains a short insertion (amino acids 329–366) absent from p35. Similar analysis of muskelin mapped the interaction with p39 to the fifth kelch repeat. Co-expression of p39 and muskelin in COS1 cells or lens epithelial cells altered the intracellular localization of muskelin, recruiting it to the cell periphery. These findings demonstrate a novel interaction between muskelin and the cyclin-dependent kinase 5 activator p39 and suggest that p39 may regulate the subcellular localization of muskelin.

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* The abbreviations used are: Cdk, cyclin-dependent kinase; EGFP (ECFP, EYFP), enhanced green (cyan, yellow) fluorescent protein; RT, reverse transcription; KREP, kelch repeat; GST, glutathione S-transferase; PBS, phosphate-buffered saline; LisH, Lissencephaly homology; CTLH, C-terminal to Lissencephaly homology; E18, embryonic day 18; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

A wide variety of other cell types, including lens (3), embryonic limb buds (4), monocytes (5), and osteosarcomas and breast carcinomas (6).

A number of observations suggest that cytoskeletal regulation may be a major function of Cdk5 in both neuronal and non-neuronal cells. For example, p39 is known to associate with the actin cytoskeleton (7). The known substrates of Cdk5/p35 include cytoskeletal proteins such as neurofilament proteins (8) and the microtubule-associated protein tau (9) as well as enzymes that regulate cytoskeletal organization such as Pak-1 (10, 11) and c-Src (12). In addition, the Cdk5 activators p35 and p39 have been shown to interact with α-actinin and Ca2+/-calmodulin-dependent protein kinase II (13), both of which have important roles in cytoskeletal organization. Finally, several studies have reported that changes in Cdk5 activity affect cytoskeletal functions such as cell motility and adhesion. For example, loss of Cdk5 activity inhibits neurite extension (14) and blocks neuronal migration during embryogenesis (15–18), whereas increases in Cdk5 activity increase substrate adhesion and retard migration in the epithelial cells of the cornea (19, 20) and lens (21).

It is not clear at present whether the same molecular mechanisms are responsible for the effects of Cdk5 in neuronal and non-neuronal cells. The wide variety of Cdk5 substrates and the likelihood that different subsets are expressed in different cell types raises the possibility that Cdk5 may exert its effects on adhesion and movement in various ways. To explore this possibility, we searched for novel interacting partners of Cdk5, p35, and/or p39 by yeast two-hybrid screening of an embryonic rat lens library. The results demonstrated that p39 interacts specifically with the kelch domain protein muskelin. Kelch domains are structural repeats first observed in the Drosophila actin cross-linking protein Kelch that permit proteins to fold into a cylindrical, “β-propeller structure” (22). Although several kelch domain proteins are also actin-binding proteins (22), muskelin does not bind directly to either actin or tubulin (23). It is, however, associated with the actin cytoskeleton and has multiple effects on cell adhesion and cytoskeletal structure in adherent cells (24).

MATERIALS AND METHODS

Yeast Two-hybrid Bait and Library Construction—The entire p39 cDNA was cloned into pBD-GAL4 Cam phagemid vector (Stratagene, La Jolla, CA). The embryonic (E18) rat lens cDNA library was constructed using 5 μg of poly(A)+ RNA cloned into hybriZAP-2.1 vector following the manufacturer’s instruction (HybriZAP-2.1 XR library construction kit and HybriZAP-2.1 XR cDNA synthesis kit; Stratagene). The primary library contained 2 × 107 plaque-forming units with an average insert length of 1 kb. Excision and amplification of the library was performed as detailed by Stratagene.

Yeast Two-hybrid Screening—YRG2 competent yeast cells were transfected with p39 bait to create a stable cell line. The YRG2/p39

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yeast cells were transiently transfected with His/Leu/Trp medium. All positive clones were further analyzed via filter lift assay screening for LacZ expression. Filter lift assays were performed by transferring colonies (growing on His/Leu/Trp SD plates (2.67% Difco™ yeast nitrogen base without amino acids (BD Biosciences, Franklin Lakes, NJ), 1 x sorbitol, 2% agar) to nitrocellulose filters, lysing cells by repetitive freeze-thaw cycles, and incubating with the β-galactosidase substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) according to the manufacturer’s protocol (Stratagene).

Plasmids from positive yeast colonies were isolated by mechanical lysis. Briefly a single yeast colony was grown overnight in 2 ml of YAPD medium (2% Difco™ peptone (BD Biosciences), 1% yeast extract, 2% agar, 0.1 M adenine sulfate, pH 5.8) at 30 °C. The yeast was centrifuged and lysed by 0.2 ml of yeast lysis buffer (2% Triton X-100, 1% SDS, 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA), 0.2 ml phenol-chloroform:isoamyl alcohol containing 0.5 M of sodium acetate and ethanol and transformed into XL-Blue MR™ competent cells. Target or bait plasmids were selected on LB-ampicillin or LB-chloramphenicol agar plates, respectively. The resulting bacterial clones were screened by restriction digest analysis and sequencing. Sequencing was performed using the CEQ dye terminator cycle sequencing (DTCS) quick start kit (Beckman Coulter). The PCR products were then digested with XhoI and XbaI. The digested p39 fragment was ligated into the pcDNA3.1/His (C) vector C-terminal to the histidine tag and in the reading frame (confirmed by PCR sequencing). The p39 truncated clone was cloned into pET15b at the NdeI and Xhol sites. The primers used were 5'-ATCCTCCGGGATCATGAGCCATGCTGTCTCTTTGCGCTGG-3' and 5'-CCGGCCGTCGGATCTTGAAGG3'-A.

To generate EGFP-p39 and EYFP-p39 fusion proteins, the full-length p39 cDNA was cloned into EcoRI/Sall sites of the pEGF-C1 or pEYFP-C1 vectors starting at the second codon of p39 using the pCDNA3.1-p39 clone as the template. The primers used were: upstream, GGCTCGAAGATCTGGACAGTCGTTCTCTCTTGTCCGCTGG (EcoRI); downstream, GGCGGTCGACCAATGGGATATCTGCTCTGTCGCTG (Sall). The resulting PCR products were cloned into the pGEX-4T-1 vector to generate GST-muskelin fusion proteins. The GST-muskelin and 35S-labeled proteins were purified from the resulting antisera using protein A-agarose beads (Pierce) and the p39 antibody was further purified by affinity chromatography against the antigenic peptide covalently coupled to agarose beads as a secondary amine using the AminoLink™ kit and AminoLink coupling gel (Pierce). Anti-Cdk5 polyclonal antibody and agarose-conjugated anti-histidine monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Myc polyclonal antibody was purchased from Cell Signaling Technology (Beverly, MA).

Specific Interaction between Muskelin and p39

Cell Culture and Transfection—The rabbit lens epithelial cell line (N/N0103A) and COS1 monkey kidney epithelial cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO2 in Dulbecco’s minimum essential medium (Invitrogen) supplemented with 8% rabbit serum, 50 μg/mg genitancim for N/N0103 cells or 10% fetal calf serum (Invitrogen), 100 mg/ml penicillin/streptomycin (Invitrogen), 1% Triton X-100, and 1% N2 medium (0.1 mM phenylmethylsulfonyl fluoride, and one Complete™ protease inhibitor mixture tablet/50 ml of buffer (Roche Diagnostics). For the detection of p39, the cells were lysed in co-immunoprecipitation buffer (50 mM Tris (pH 7.5), 150 mM EGTA, 100 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and Complete™ protease inhibitor), and the remaining pellet, the insoluble fraction, was resuspended in PBSTDS. Both fractions were immunoprecipitated using the Cdk5 monoclonal antibody (J-3, Santa Cruz Biotechnology).

Intracellular interactions were shown by transiently transfecting Myc-muskelin into COS1 cells or COS1 cells that stably expressed p39. Seventy-two hours post-transfection, cells were lysed with immunoprecipitation buffer (50 mM Tris (pH 7.5), 150 mM EGTA, 100 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) plus Complete™ protease inhibitor. The lysates were immunoprecipitated with agarose-conjugated anti-histidine monoclonal antibody (Santa Cruz Biotechnology) and resolved on a NuPage 4–12% BisTris gel (Novex). Immunoblotting was performed as described previously (3). The bands were visualized using a chemiluminescent substrate (Pierce), and the pl39 antibody was further purified by affinity chromatography against the antigenic peptide covalently coupled to agarose beads as a secondary amine using the AminoLink™ kit and AminoLink coupling gel (Pierce). Anti-Cdk5 polyclonal antibody and agarose-conjugated anti-histidine monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

GST Fusion Proteins and Affinity Purification Pull-down Assay—To express the various pEGF-C1-true muskelin clones a 100-ml culture was inoculated and incubated at 30 °C overnight. The following day the culture was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside for 3 h and harvested at 5000 rpm. The pellet was resuspended in 3 ml of 1× PBS plus protease inhibitors and lysosome to 0.1 volume. Samples were kept on ice for 30 min, adjusted to 5 mM dithiothreitol, and sonicated. The sonicate was adjusted to 1% Triton X-100, incubated at room temperature on a rotary wheel for 30 min, and centrifuged at 12,000 rpm. The supernatant was added to gluthatione-coupled beads and processed according to the manufacturer’s protocol (Amersham Biosciences). 1 μg of GST-muskelin was used per experiment. To generate 35S-labeled p39, p35, Cdk5, and muskelin, the corresponding cDNAs were cloned into pcDNA3.1 (for p39, p35, and Cdk5) (Invitrogen) or pcDNA3.1/His (for muskelin). The clones were transfected into HEK293 cells (San Diego, CA) and translated in vitro using the TNT quick coupled transcription/translation system (Promega, Madison, WI). The GST-muskelin and 35S-labeled proteins were incubated overnight in the immunoprecipitation buffer (above) containing 1 mg of BL21 soluble protein extract. The following day the bands were washed extensively in immunoprecipitation buffer. The proteins were eluted with SDS loading buffer (Invitrogen) and resolved on a NuPage 4–12% BisTris gel. The gels were stained with Coomassie
DNA binding domain and used as baits for yeast two-hybrid screening of an E18 embryonic rat lens cDNA library. With the GAL4 DNA binding domain/p39 (pBD-p39) fusion plasmid as bait we isolated several prey sequences that supported growth on medium lacking histidine, leucine, and tryptophan and had significant β-galactosidase activity. Sequence analysis of -57 such plasmids after rescue in Escherichia coli yielded α-crystallins (4), β-crystallins (12), γ-crystallins (17), a cytoskeletal protein (1), a hypothetical pvoxirxin and zinc finger (POZ) domain protein (1), and the nearly complete sequence of muskelin cDNA (pAD-muskelin), lacking only the first nine bases. Muskelin encodes a polypeptide of 735 amino acids containing six kelch motifs, a discoidin domain and a LisH motif, and a CTHL domain (24) (Fig. 2A).

The specificity of the interaction between pAD-muskelin and p39 was examined by co-transforming yeast with this clone in conjunction with pBD-p39, pBD-p35, pBD-Cdk5, or the BD fusion of an unrelated protein, human lamin C. Only the muskelin/p39 co-transformants grew in the absence of leucine, tryptophan, and histidine (Fig. 2B) and activated LacZ transcription in a filter lift assay (not shown).

In Vitro Interaction between Muskelin and p39—To confirm the interaction between muskelin and p39 detected by the yeast two-hybrid analysis, a complete muskelin cDNA was constructed by PCR and cloned into a plasmid to generate a fusion protein with a GST tag at the C terminus. GST-muskelin was immobilized on a glutathione-agarose matrix and incubated with in vitro translated 35S-labeled p39, p35, or Cdk5 (Fig. 3, A and B). The proteins retained on the matrix were eluted, analyzed by SDS-PAGE, dried, and exposed to film. Consistent with the yeast data, the GST-muskelin fusion protein interacted with p39 but not with p35 or Cdk5 (Fig. 3, A and B).

To determine which region of p39 was recognized by muskelin, we performed a similar GST pull-down experiment with a chimeric fusion protein that links the N terminus of p35 with the C terminus of p39, the isolated p39 C terminus (amino acids 110–367), and a p39 truncation lacking the C terminus (amino acids 1–328) (Fig. 3A). GST-muskelin bound to the p39 C terminus and the p55/p39 chimera but not to the p39 truncation, thus localizing the muskelin binding site to the C-terminal end of the p39 protein (amino acids 329–367) (Fig. 4).
lated, 35S-labeled proteins. GST-muskelin bound to 35S-labeled p39, the and immunoblotted with rabbit polyclonal anti-Myc antibody to detect His-Cdk5, His-p35, or His-p39. Cell lysates (amino acids at the C terminus (amino acids 1–328).

Intracellular Interaction between Muskelin and p39—We next examined the ability of muskelin and p39 to interact in mammalian cells. Myc-tagged muskelin cDNA was transiently transfected into COS1 cells or COS1 cells that had been stably transfected with His-Cdk5, His-p35, or His-p39. Cell lysates (L) were immunoprecipitated with mouse monoclonal antibody against the histidine tag and immunoblotted with rabbit polyclonal anti-Myc antibody. Immunoblotting demonstrated that Myc-muskelin co-immunoprecipitated with histidine-tagged proteins from His-p39-transfected cells but not from cells transfected with His-p35 or His-Cdk5 (Fig. 3C). Thus, muskelin appears to interact specifically with p39 both in vitro and in an intracellular environment.

Mapping of Interaction Sites in Muskelin—To establish which domain of muskelin was important for binding to p39, we generated a series of GST-muskelin deletions, which sequentially removed each of the six kelch domains (Fig. 4A and B). Each GST-muskelin construct was then incubated with in vitro translated 35S-labeled p39 (Fig. 4C). Deletion of the C terminus and sixth kelch domain had little effect on the ability of muskelin to bind p39. In contrast, loss of the fifth kelch domain caused a significant loss of the muskelin-p39 binding, suggesting that this region plays an important role in binding p39. Subsequent deletion of the third and fourth kelch domains slightly increased binding but not to the levels seen with the intact protein. The N-terminal portion of the protein (amino acids 1–204), which contains the discoidin and LisH domains, showed little affinity for p39 (Fig. 4C), underscoring the central importance of the β-propeller region.

Because removal of the fifth kelch domain was expected to disrupt the β-propeller structure, an additional experiment was performed to determine whether binding to p39 requires specific sequences within the fifth domain or merely an intact β-propel-
For this, we generated a construct that replaces the loop region of the fifth kelch domain with an equal number of residues from the loop region of the sixth domain. Although this six-domain construct has the structural features necessary to reconstitute the β-propeller, it did not restore binding to p39 (Fig. 4D). Thus, specific amino acid sequences within the fifth kelch domain of muskelin seem to be necessary for the interaction.

**Endogenous Muskelin Expression in Cells and Tissues**—Endogenous expression of muskelin mRNA was examined by RT-PCR of RNA extracted from embryonic (E18) rat brain and lens (Fig. 5A). A single PCR product of the expected size was present in samples in the presence of reverse transcriptase. Sequencing of this product demonstrated that it was derived from muskelin mRNA. To detect endogenous expression of muskelin protein, protein extracts from E18 rat brain and lens were immunoblotted with anti-muskelin antibody. This experiment also included protein extracts from C2C12 myoblasts, which have previously been shown to express muskelin (24), and COS1 cells, which we used for transfection studies (see below). Immunoblotting detected an immunoreactive band with an apparent molecular weight of ~80,000 in all four samples (Fig. 5B) in good agreement with the predicted molecular weight of muskelin (84,833). The immunoreactivity of this band was blocked by the presence of the antigenic peptide, confirming the specificity of the reaction. A more rapidly migrating band observed in C2C12 and lens fibers cells was apparently nonspecific as it was not competed by the antigenic peptide (Fig. 5B, right panel). Finally we tested whether endogenous muskelin interacts with p39 in lens and brain. Protein extracts from E18 rat lens and brain were immunoprecipitated with anti-p39 antibody and then immunoblotted for muskelin. Muskelin and p39 were co-immunoprecipitated from both tissues, indicating that they are part of an in vivo protein complex (Fig. 5C). No muskelin was detected in control experiments lacking the p39 antibody (Fig. 5C).

**In Vivo Expression of Muskelin in the Lens and Other Tissues**—To examine the in vivo expression pattern of muskelin in the lens, we first performed immunofluorescence on paraffin sections of adult (2-month-old) mouse eye (Fig. 6, A–D). Specific staining was seen in the outer cortical fiber cells of the lens, the lens epithelium, and many other ocular tissues, including the corneal epithelium, retina, and ocular muscles (Fig. 6, A and B). Immunofluorescence was particularly intense near the posterior tips of the elongating fiber cells, which are in contact with the lens capsule (Fig. 6, A and D), and often seemed to be arrayed along cell-cell boundaries (Fig. 6C).

To confirm that muskelin was also expressed during development and to examine its localization pattern, we performed immunohistochemistry on cranial sections of E17–18 embryonic rats. At this stage of development even the primary fiber cells at the center of the lens are not yet fully differentiated (29). Specific immunostaining was seen throughout the lens with the most intense staining in the elongating lens fiber cells near the lens equator and in the posterior tips of the fiber cells (Fig. 7). Muskelin expression was also seen in the neural precursor cells of the retina, parts of the central nervous system, and epithelial tissues (including the nasal epithelium and epidermis) (Fig. 7). Interestingly many regions with high levels of muskelin expression, including the retina, trigeminal ganglia,
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**Fig. 7. Muskelin expression in E17-18 rat cranial section.** A, immunohistochemistry showing positive staining for muskelin in lens (L), retina (R), optic nerve (OpN), inferior olivary nucleus (ON), trigeminal ganglion (TG), facial ganglion (FG), nasal epithelium (NE), and epidermis (E). B, control sections incubated in parallel in the presence of the antigenic peptide.

**Fig. 8. Immunofluorescence of muskelin (left column) and p39 (right column) in co-transfected cells.** A, immunofluorescence of a COS1 cell transfected with ECFP-muskelin only. The cell has well spread morphology, and muskelin is evenly distributed throughout the cell interior or in small puncta. B, a cell in the same field expressing both ECFP-muskelin and EYFP-p39. Expression of fusion proteins of the correct size was confirmed by immunoblotting (not shown). COS1 cells that were transiently transfected with muskelin alone showed diffuse cytoplasmic localization with numerous small puncta (Fig. 8A). This localization was seen in all cells examined (26 of 26 cells imaged) and resembles the reported localization of muskelin in other cell types (23, 24). Cells that were transfected with p39 alone showed p39 accumulation in the nucleus if expression levels were high. Such cells were often rounded and showed signs of toxicity (not shown). However, in cells with lower levels of p39 expression, p39 localized along cell margins and in diffuse plaques (8 of 8 cells imaged) (Fig. 8D). A similar localization of p39 was seen previously in transfected COS7 cells (7). Co-transfection of p39 and muskelin led to a marked relocalization of muskelin and extensive co-localization with p39 in the perinuclear region, in lamellipodia, and along the cell periphery (8 of 11 cells imaged) (Fig. 7, E and F). Closer examination of the co-localization of p39 and muskelin in co-transfected COS1 cells showed a preferential association of both proteins with concave cell edges (19 of 33 concave edges) as compared with convex cell edges (4 of 31 convex edges), suggesting a possible role in contraction.

**Fig. 7**

**Fig. 8**

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facial ganglia, and inferior olivary nuclei, have previously been reported to express p39 (30, 31).

**p39 Alters the Subcellular Localization of Muskelin—**To examine the functional consequences of the p39-muskelin interaction, COS1 cells were transiently transfected with ECFP-muskelin and EYFP-p39. Expression of fusion proteins of the correct size was confirmed by immunoblotting (not shown). COS1 cells that were transiently transfected with muskelin alone showed diffuse cytoplasmic localization with numerous small puncta (Fig. 8A). This localization was seen in all cells examined (26 of 26 cells imaged) and resembles the reported localization of muskelin in other cell types (23, 24). Cells that were transfected with p39 alone showed p39 accumulation in the nucleus if expression levels were high. Such cells were often rounded and showed signs of toxicity (not shown). However, in cells with lower levels of p39 expression, p39 localized along cell margins and in diffuse plaques (8 of 8 cells imaged) (Fig. 8D). A similar localization of p39 was seen previously in transfected COS7 cells (7). Co-transfection of p39 and muskelin led to a marked relocalization of muskelin and extensive co-localization with p39 in the perinuclear region, in lamellipodia, and along the cell periphery (8 of 11 cells imaged) (Fig. 7, E and F). Closer examination of the co-localization of p39 and muskelin in co-transfected COS1 cells showed a preferential association of both proteins with concave cell edges (19 of 33 concave edges) as compared with convex cell edges (4 of 31 convex edges), suggesting a possible role in contraction.

To test whether p39 produces similar effects on muskelin localization in lens epithelial cells, N/N1003 lens epithelial cells were transfected with Myc-muskelin and/or EGFP-p39. Expression of both fusion proteins was confirmed by immunoblotting (not shown). The localization patterns seen in lens epithelial cells closely resembled those seen in COS1 whether the cells were transfected with each fusion construct separately (not shown) or with both together (Fig. 7, G and H). Again co-expression of p39 and muskelin directed muskelin to sites along the cell periphery.

To determine whether these peripheral sites were associated with the actin cytoskeleton, lens epithelial cells that had been co-transfected with Myc-muskelin alone (Fig. 9, A–C) or EGFP-p39 and Myc-muskelin (Fig. 9, D–F) were co-stained with rhodamine-phalloidin. In lens epithelial cells that were transfected with muskelin only, muskelin was diffusely localized in the cytoplasm (Fig. 9B) and showed no association with cortical actin cytoskeleton (Fig. 9C). The cytoskeletal organization in these cells was indistinguishable from that in non-transfected cells in the same field (Fig. 9C). In contrast, lens epithelial cells that were doubly transfected with muskelin and p39 showed localization of both proteins along portions of the peripheral actin cytoskeleton (Fig. 9,
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D–F, arrow). The regions where muskelin and p39 associated with the cytoskeleton again appeared to be primarily along the trailing edges of the cell, and the cortical actin cytoskeleton itself was not noticeably altered.

DISCUSSION

The present study demonstrates that the Cdk5-activating protein p39 interacts with muskelin, a kelch domain protein that is widely expressed during development. Muskelin is an intracellular protein of unknown function first isolated on the basis of its ability to promote cell adhesion to the C-terminal domain of thrombospondin 1 (24). When overexpressed, muskelin also affects the formation and dynamics of focal adhesions in cells grown on fibronectin (24). The multidomain structure of muskelin suggests that it may participate in numerous protein-protein interactions. Indeed muskelin has been shown to interact with the prostaglandin receptor EP3 (32) and the high molecular weight Ran-binding protein (RanBPM) (33) in addition to the interaction with p39 reported here. The muskelin binding site on p39 maps to a unique insertion in the p39 C terminus. The ability of muskelin to interact specifically with p39 at a site not present in p35 is consistent with recent findings indicating that p35 and p39 may have different intracellular substrates (34), distinct subcellular localizations (35), and independent functions (35) under physiological conditions.

Interestingly a previous study has shown that the N-terminal region of p39 is able to bind directly to the actin cross-linking protein α-actinin (13). Because the interaction between p39 and muskelin involves the C-terminal region of p39, the present findings raise the possibility that p39 may tether muskelin to α-actinin and thus to the actin cytoskeleton. In support of this possibility, we found that p39 prompts a marked rearrangement of muskelin in co-transfected cells, leading to co-localization of both proteins at sites along the peripheral actin cytoskeleton. Moreover because the regions of p39 that interact with α-actinin and muskelin are outside the Cdk5 binding region (13, 36), p39 may recruit Cdk5 into a multiprotein complex containing muskelin, α-actinin, and the actin cytoskeleton. In support of this possibility, muskelin is most concentrated near the posterior tips of the fiber cells where they contact the lens capsule, a region where Cdk5 (21) and cytoskeletal proteins (37) are also highly concentrated. The strong similarity between the previously reported co-localization of Cdk5 and p39 along the periphery of transfected COS7 cells (7) and the peripheral co-localization of muskelin and p39 found in the present study further support the possibility of a Cdk5-p39-muskelin complex associated with the peripheral actin cytoskeleton. Assembly of such a complex would be consistent with the reported effects of both muskelin (24) and Cdk5 (14, 20, 21, 38) on cytoskeletal organization, adhesion, and migration.

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Fig. 9. Co-localization of p39 and muskelin with the actin cytoskeleton. N/N1003A lens epithelial cells were transfected with Myc-muskelin only or co-transfected with EGFP-p39 and Myc-muskelin and then stained with rhodamine-phalloidin to detect the actin cytoskeleton. A, negative control. No immunofluorescence was detected in the green channel in cells that were transfected with Myc-muskelin alone. B, cells that were singly transfected with Myc-muskelin showed diffuse muskelin immunofluorescence throughout the cytoplasm. C, the actin cytoskeleton in the transfected cells showed normal and was unrelated to the distribution of muskelin. D, EGFP fluorescence detects two cells with different levels of EGFP-p39 expression. E, Alexa350 immunostaining of anti-Myc antibody was used to detect Myc-muskelin in doubly transfected cells. Myc-muskelin co-localized with p39 at the cell periphery (arrow). Co-localization of EGFP-p39 and Myc-muskelin was also observed in the upper cell in this field when the image intensity was enhanced. F, the same field stained with rhodamine-phalloidin to reveal the actin cytoskeleton. Localization of p39 and muskelin along the cell periphery coincided with cortical actin staining (arrow). Scale bar, 50 μm.
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