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# Calmodulin-like Protein Increases Filopodia-dependent Cell Motility via Up-regulation of Myosin-10*

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**Human calmodulin-like protein (CLP) is an epithelial-specific protein that is expressed during cell differentiation but down-regulated in primary cancers and transformed cell lines. Using stably transfected and inducible HeLa cell lines, we found that CLP expression did not alter the proliferation rate and colony-forming potential of these cells. However, remarkable phenotypic changes were observed in CLP-expressing compared with control cells. Soft agar colonies of CLP-expressing cells had rough boundaries, with peripheral cells migrating away from the colony. Cells expressing CLP displayed a striking increase in the number and length of myosin-10-positive filopodia and showed increased mobility in a wound healing assay. This increase in wound healing capacity was prevented by small interference RNA-mediated down-regulation of myosin-10. Fluorescence microscopy and Western blotting revealed that CLP expression results in up-regulation of its target protein, myosin-10. This up-regulation occurs at the protein level by stabilization of myosin-10. Thus, CLP functions by increasing the stability of myosin-10, leading to enhanced myosin-10 function and a subsequent increase in filopodial dynamics and cell migration. In stratified epithelia, CLP may be required during terminal differentiation to increase myosin-10 function as cells migrate toward the upper layers and establish new adhesive contacts.**

Human calmodulin-like protein (CLP)[2] was first discovered as the product of an intronless gene expressed in normal epithelial cells but strongly down-regulated in transformed cells and epithelial cancers (1–3). Like calmodulin (CaM), CLP contains four "EF-hand" $Ca^{2+}$ binding motifs but has an overall $Ca^{2+}$ binding affinity that is ~10-fold lower than in CaM (4). The x-ray crystal structure indicates that $Ca^{2+}$-CLP is very similar in overall shape to $Ca^{2+}$-CaM, although a notable difference is observed in the angle of the central helix with respect to the N- and C-terminal lobes. Major differences are also apparent in the putative target-interacting surfaces of the two lobes, including the hydrophobic patches thought to be involved in target recognition (5). Accordingly, although CLP can substitute for CaM in binding and activation of some targets (*e.g.* CaM kinase II), many CaM targets either bind CLP with reduced affinity or not at all (4, 6). On the other hand, recent data have shown that CLP interacts with unique targets, specifically with the unconventional myosin Myo-10 (7). This suggests that CLP has a physiological function distinct from that of CaM and that CLP modulates a subset of CaM-regulated proteins and/or interacts with its own specific targets.

CLP is exclusively expressed in normal pseudostratified and stratified epithelia such as those of breast, prostate, and skin (3, 8). The developmental- and growth factor-dependent patterns of expression further suggest that CLP is involved in the terminal differentiation process of multilayered epithelia (9), but its physiological role remains unknown. To study the effect of CLP in a cellular environment, we generated tetracycline-inducible stable HeLa cell lines. We here report that CLP expression does not change the cell proliferation rate and does not affect the colony-forming potential or the colony size of cells growing in soft agar. However, colony morphology was altered, with edges being irregular and indicative of increased peripheral cell motility. CLP expression resulted in increased cell motility in a wound healing assay, and individual cells displayed more and longer filopodia with increased levels of Myo-10. Biochemical analysis revealed that CLP up-regulates Myo-10 by increasing its intracellular stability. These findings show that CLP function is mechanistically linked to Myo-10 and suggest that CLP promotes cell motility by prolonging Myo-10 function. In stratified epithelia, CLP function may be required during migration and/or adhesion of terminally differentiating cells.

## EXPERIMENTAL PROCEDURES

*Materials*—All cell culture media and reagents (trypsin-EDTA, Dulbecco's modified Eagle's medium, fetal bovine serum, L-glutamine, sodium pyruvate, and antibiotics/antimycotics) were from Invitrogen. PCR reagents, enzymes, and protease inhibitor mixture tablets (Complete mini EDTA-free) were purchased from Invitrogen. All other chemicals were from Sigma. X-ray films were from Eastman Kodak.

*Plasmid Constructions*—Construct pEGFP-Myo10 was made by cloning the full-length human Myo-10 coding sequence as KpnI-XbaI fragment into pEGFP-HA-His (7). Construct pEGFP-Myo10-(1–980) was made by cloning a PCR fragment of Myo-10 cDNA (GenBank™ accession number AF234532) corresponding to nucleotides 223–3165 into pEGFP-

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[2] The abbreviations used are: CLP, calmodulin-like protein; CaM, calmodulin; DPBS, Dulbecco's phosphate-buffered saline; GFP, green fluorescent protein; EGFP, enhanced GFP; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Myo-10, myosin-10; PIPES, 1,4-piperazinediethanesulfonic acid; CM, $Ca^{2+}/Mg^{2+}$; dox, doxycycline; siRNA, small interference RNA.



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HA-His. The PCR product was generated using primer Myo10F2, 5′-GTA GGT ACC ATG GAT AAC TTC TTC ACC GA-3′, containing an added KpnI site (underlined) and primer Myo10–3165R, 5′-GTT TCT AGA TGT TGG GCT TCT CCT CGC-3′ with an added XbaI site (underlined). This fragment was A-tailed using Klenow fragment (Invitrogen) cloned into the TA-TOPO cloning vector (Invitrogen), excised using KpnI/XbaI double digestion, and then ligated into pEGFP-HA-His that had been linearized with KpnI and XbaI. To create a construct for tetracycline-inducible expression of CLP, the open reading frame of CLP was first PCR amplified using primers CLP-forward, 5′-AAG CTT CAC CCC TGG CAT-3′, (added HindIII site underlined) and CLP-reverse, 5′-CTC GAG GCC TCA CTT GG-3′ (added XhoI site underlined). The resulting fragment was TA cloned, excised by HindIII/XhoI double digestion, and ligated into HindIII/XhoI-digested pcDNA4/TO (Invitrogen) to generate construct pcDNA4/TO-CLP. All constructs were verified by nucleotide sequencing in the Mayo Genomics Core facility.

*Antibodies*—Affinity-purified rabbit polyclonal antibody TG7 against human CLP has been described (8). An affinity-purified polyclonal antibody (MX-117) against bovine Myo-10 HMM was a generous gift from Dr. Richard Cheney (University of North Carolina, Chapel Hill) and has been described (10). Monoclonal antibodies against β-actin and GFP were from Sigma and Clontech (Palo Alto, CA), respectively, and monoclonal anti-GAPDH antibodies were from RDI (Concord, MA). Rhodamine-phalloidin and all secondary antibodies for immunofluorescence were from Invitrogen. Secondary antibodies for Western blotting were from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal antibody against human Myo-10 was generated by immunizing rabbits with a synthetic peptide corresponding to residues 936–953 of the protein (NCBI accession number AAF37875). The peptide (RAAQEFLESLNFDEIDEC) was synthesized in the Mayo Proteomics Core and attached via its C-terminal Cys residue to keyhole limpet hemocyanin before use as antigen. Antisera were raised in two rabbits maintained and treated at Cocalico, Inc. (Reamstown, PA). Antibodies were affinity purified by chromatography over Sepharose coupled to a fusion protein of the antigenic peptide with bovine serum albumin. Affinity-purified antibodies were stored at a concentration of ~0.15 μg/μl.

*Cell Cultures and Transfections*—HeLa cells stable for the expression of the tetracycline repressor protein were obtained from Invitrogen and maintained in Dulbecco's modified Eagle's medium containing high glucose, L-glutamine, 110 mg/liter sodium pyruvate with pyridoxine hydrochloride (11995-065; Invitrogen), as well as 10% fetal bovine serum and 50 μg/ml G418 (to maintain Tet-repressor expression). Control HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in minimal essential medium with balanced Earle's salts and L-glutamine, non-essential amino acids, and 110 mg/liter sodium pyruvate (11095-080; Invitrogen), and supplemented with 10% fetal bovine serum. All cell lines were maintained with antibiotic/antimycotic (Invitrogen) and cultured at 37 °C in a humidified atmosphere containing 5% $CO_2$. For immunolocalization and fluorescence microscopy, cells were grown on glass coverslips in 12-well plates. 50–70% confluent cells were transfected with 1 μg of plasmid DNA/6-cm dish using Lipofectin™ and Plus™ (Invitrogen) following the manufacturer's recommended protocols.

*Generation of Stably Transfected Cell Clones*—HeLa cells were transfected with the CLP expression vector pcDNA4/TO-CLP, and stable transfectants were selected in medium containing Zeocin (3 μg/ml) as recommended by the supplier of the T-REx system (Invitrogen). Control clones were established by transfection with an empty pcDNA4/TO vector. After 6–10 days in selection medium, surviving clones were isolated using cloning cylinders and expanded for storage and further analysis. Clones were tested for the induction of CLP in the presence of 0.25 μg/ml doxycycline or tetracycline (Sigma). Cells in passages 8–20 were used in this study.

*Preparation of Cell Lysates and Western Blotting*—Cells in culture dishes were rinsed twice with cold $Ca^{2+}$-/$Mg^{2+}$-free phosphate-buffered saline (DPBS; Invitrogen) containing a protease inhibitor mixture (aprotinin, leupeptin, pefabloc, and pepstatin). Cells were scraped, pelleted, and used immediately or stored at −80 °C until further use. Cell extracts were prepared by resuspending the pellets in a buffer containing 50 mM HEPES at pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitors and lysed by pipetting up and down on ice. The samples were centrifuged at 4 °C for 15 min at 13,000 × *g*. Protein concentrations were measured spectrophotometrically using the BCA assay (Pierce). Appropriate amounts of protein were mixed with NuPAGE electrophoresis buffer in the presence of reducing agents and antioxidants and heated to 70 °C for 15 min, followed by separation in denaturing 4–12% NuPAGE gradient gels following the recommendations of the supplier (Invitrogen). Western blotting was performed according to standard procedures (11) or following a protocol (12) to improve the detection of CLP (9). The membranes were blocked for 1 h at room temperature in 5% nonfat milk in CaTBST (2 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) prior to incubation for 1.5 h at room temperature or overnight at 4 °C in primary antibody diluted in CaTBST plus 5% nonfat dry milk. The following primary antibodies were used: TG7 (anti-CLP) at 0.2 μg/ml, MX-117 (anti-bovine Myo-10) at 1 μg/ml, Myo10 2-2 (anti-human Myo-10) at 1:2,000, and anti-GFP at 1:2,000. In addition, some blots were reprobed with an anti-β-actin antibody (1:1,000) or anti-GAPDH (1:3,000) as a housekeeping protein marker to ensure equal protein loading. Following exposure to primary antibodies, the blots were rinsed in CaTBST, incubated for 1 h in horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (1:5,000), rinsed again in CaTBST, and processed using the ECL-Plus detection system (Amersham Biosciences).

*Semiquantitative Reverse Transcription PCR of Myo-10 mRNA*—Total RNA was prepared from control and CLP-expressing HeLa cells using the RNAeasy mini spin kit from Qiagen according to the manufacturer's protocol for mammalian cells grown in a monolayer. cDNA was generated using 1 μg of total RNA and random hexamer oligonucleotides and Superscipt III (Superscript III First-Strand Synthesis System for reverse transcription PCR; Invitrogen). PCR was performed using 1 μl of cDNA in a 25-μl volume containing 5 pmol each of

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specific Myo-10 primers Myo10–2102F (5′-GCT CCT CTA ATC CTT TCT T-3′) and Myo10–2675R (5′-TTG CTC CCT TTC TCT GC-3′) or primers specific for β-actin (sense 5′-GCT CGT CGT CGA CAA CGG CTC-3′ and antisense 5′-CAA ACA TGA TCT GGG TCA TCT TCT C-3′) (Invitrogen), 1 μl of 10 mM dNTPs, 1.5 μl of 50 mM $MgCl_2$, and *Taq* polymerase in 1× reaction buffer (Invitrogen). Touchdown PCR was done by 1 min of denaturation at 94 °C followed by annealing for 60 s at 66 °C and extension at 72 °C for 90 s. Annealing temperature was lowered by 2 °C for 5 cycles, and 15 to 35 additional cycles were then performed to determine the linear phase of amplification. After a final extension of 5 min, the samples were cooled to 4 °C and aliquots run on a 1% agarose gel stained with ethidium bromide.

*Cell Proliferation Assay*—Control and CLP-expressing cells were plated at $10^3$ cells/well on 96-well plates and grown under standard conditions in the continued presence of selective antibiotics and of 0.25 μg/ml doxycycline (as appropriate). Samples were counted every day for 3–4 days using a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay (ACTC) according to the manufacturer's instructions and optimized for these cell types. At the time of assay, 100 μl of tetrazolium salt reagent (as supplied) was added to each well and incubated for 30 min under standard cell culture conditions (37 °C, 5% $CO_2$). The reaction was halted by the addition of 200 μl of extracting reagent (as supplied) and incubated overnight in the dark. The samples were then analyzed using a microplate (Molecular Devices, Sunnyvale, CA) reader at 600 nm. The $A_{600}$ values determined for each sample were then compared with a standard curve for each cell type, and cell numbers/well were calculated and plotted as a function of the number of days in culture ± S.D.

*Colony-forming Assay*—To assay for contact-independent growth in soft agar, 10-cm gridded Petri dishes were first coated with 1.5 ml of a base agar consisting of 0.5% agar B (Difco) in culture medium (Dulbecco's modified Eagle's medium with high glucose, 110 mg/liter sodium pyruvate, 10% fetal bovine serum, sodium bicarbonate, and antibiotic/antimycotic). Control and CLP-expressing cells (± doxycycline) were trypsinized, counted using a hemocytometer, and then resuspended at low density (5,000 cells/1.5 ml) in 0.35% agar B in culture medium with or without doxycycline kept at ~40 °C. After pouring into the 10-cm dishes containing base agar, the immobilized cells were grown for 14 days in a humidified chamber at 37 °C with 5% $CO_2$. Plates were stained with 0.005% crystal violet in phosphate-buffered saline for 1 h. Colonies were then photographed at low magnification (×10 objective) using a side port-mounted Sensi-Cam QE CCD camera (Cooke Corp., Auburn Hills, MI) and were then counted and measured using IP-Lab software.

*Wound Healing Assay*—Cells were grown to confluence on gridded plastic dishes, and monolayers were then wounded by scratching with a 10-μl pipette tip. Inducible HeLa cells were preincubated for 24 h with doxycycline to induce maximal CLP expression before wounding. Wounds were photographed under the microscope (×20 objective) and their coordinates recorded. The same wounds were photographed again 8 h later. The area of the wound covered by cells was measured using IP-Lab software. Briefly, a region of interest was drawn to encompass the wound area free of cells. This area was calculated for at least eight independent wounds on three separate plates at times 0 and 8 h post-wounding with and without induced CLP expression. Cell migration into the wound was then calculated as the area of the wound covered after 8 h on each side of the wound ((area at 0 h − area at 8 h)/2).

*Immunofluorescence Confocal Microscopy*—Untransfected and transiently transfected cells grown on glass coverslips for 24–72 h were washed with warm DPBS + $Ca^{2+}/Mg^{2+}$ (DPBS + CM; Invitrogen) and fixed for 15 min at 37 °C in 0.1 M PIPES, 1.0 mM EGTA, 3.0 mM $MgSO_4$, and 4% paraformaldehyde (Tousimis, Rockville, MD) diluted in DPBS + CM, pH 6.95. Following three 3-min washes in DPBS + CM, cells were permeabilized in 0.1% Triton-X in DPBS + CM for 2 min. After washing with DPBS + CM, the cells were blocked for 1 h at 37 °C in DPBS + CM containing 5% normal goat serum, 15% bovine serum albumin, 5% glycerol, and 0.04% sodium azide (blocking buffer) and were then incubated for 1 h at 37 °C with the appropriate primary antibody diluted in blocking buffer. Anti-Myo10-HMM MX-117 was used at 5 μg/ml. After washing three times for 10 min in DPBS + CM, the cells were incubated for 1 h at 37 °C in darkness with the appropriate secondary antibody (1 μg/ml Alexa-488 goat anti-rabbit IgG; Molecular Probes, Eugene, OR) and 25 ng/ml rhodamine-phalloidin diluted in blocking buffer. To stain nuclei, 4′,6′-diamidino-2-phenylindole dihydrochloride (Molecular Probes) was also added to the secondary antibody application at 20 μg/ml. Following incubation, cells were washed three times for 10 min with DPBS + CM and once with $dH_2O$, and coverslips were mounted onto slides using Prolong mounting medium (Invitrogen). Confocal micrographs were taken with a Zeiss LSM 510 and captured using Zeiss LSM 510 software. Images were imported and edited using Adobe Photoshop.

*Filopodia Measurements*—Filopodia were documented under epifluorescence illumination on low density cultures of live cells expressing GFP-tagged Myo-10 or of fixed cells stained for indirect immunofluorescence with antibody against Myo-10. To determine the number of filopodia, all filopodial extensions longer than 2 μm were counted on 10 individual cells/sample, and the average/cell was calculated ± S.E. To calculate the average filopodial length, the distance from the cell base to the tip was determined for all filopodia (>2 μm) of at least three separate cells in each sample (a total of 100 filopodia were analyzed/sample), and the average calculated per filopodium ± S.E.

*Myosin-10 Down-regulation by siRNA*—Three Stealth™ siRNAs specific to Myo-10 and a control siRNA were purchased from Invitrogen (MYO10-HSS106909, MYO10-HSS106910, MYO10-HSS106911, and P/N 46–2002) and were transfected into HeLa cells using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's recommended protocols. Briefly, cells were seeded in 6-well plates or gridded 10-cm dishes in 2 ml of regular growth medium without any antibiotics so the cells would be 50–60% confluent at the time of transfection. For transfection, 100 pmol of siRNA oligomer were diluted in 250 μl of Opti-MEM™ (Invitrogen). 5 μl of Lipofectamine™ 2000 were diluted in 250 μl of Opti-MEM™ and incubated for 5 min at room temperature before mixing

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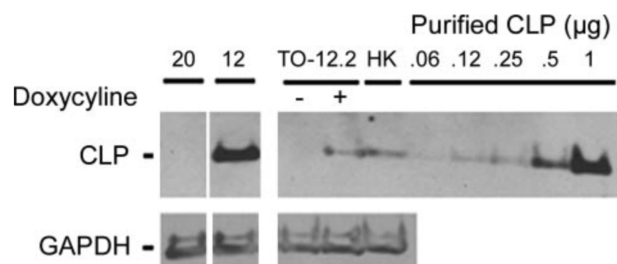


FIGURE 1. **Characterization of CLP expression in stably transfected HeLa cell lines.** Aliquots of total cell lysates from selected HeLa cell lines (indicated on *top of the lanes*) and from 4-day cultures of human keratinocytes (*HK*) as control were separated by SDS-PAGE and tested for CLP expression by Western blotting using anti-CLP antibody TG7. Cells from inducible HeLa clone TO-12.2 were grown without or with 1 $\mu$g/ml doxycycline as indicated on *top of the lanes*. Known amounts of purified CLP were run as standards for quantification of the CLP expression level in the cell lines. The blot of the cell lysates was reprobed for GAPDH to control for equal protein loading (*bottom panel*).

with the diluted siRNA. The siRNA-Lipofectamine™ 2000 mixture was incubated for 20 min at room temperature and then added to the cells. After 8 h of incubation, the medium was replaced with growth medium containing antibiotics. Cells were then assayed 24–48 h post-transfection (wound healing followed by Western blotting).

## RESULTS

*Expression of CLP Does Not Change the Proliferation Rate of HeLa Cells*—CLP is down-regulated or absent in transformed cells, suggesting its expression may be incompatible with the transformed phenotype. We used the inducible T-REx system to generate stable HeLa cell clones in which CLP expression is repressed in the absence of tetracycline (or its analog doxycycline) but can be turned on upon addition of the inducing agent. Several clones were selected for further study (Fig. 1). Interestingly, one clone (clone 12) expressed CLP constitutively even in the absence of tetracycline and maintained this property through repeated cell passages. The amount of CLP expression in the stable HeLa clones was compared with endogenous levels of CLP in human keratinocytes and to a standard curve of purified CLP as shown in Fig. 1. Clone 12 expressed the highest amount of CLP (~3% of the total soluble protein), more than 3-fold the level in 4-day keratinocytes. The other clones (*e.g.* clone TO12.2, see Fig. 1) expressed a more moderate amount of CLP comparable with the amounts in normal non-differentiating human keratinocytes.

To determine the effects of CLP expression on the phenotype of HeLa cells, we performed a series of assays with several independent stable and inducible clones, which yielded comparable results for each cell line. Growth curves were first established for stable transfected control cells that do not express CLP (clone 20) and cells that express CLP at very high levels (clone 12). As shown in Fig. 2*A*, no significant difference was seen in the proliferation rate of control and CLP-expressing cells. Similarly, no difference in proliferation rate was seen in inducible HeLa clones grown in the absence (no CLP expression) or presence (CLP expressed) of doxycycline (Fig. 2*B*). Thus, HeLa cells expressing CLP proliferate normally and at rates similar to their non-expressing counterparts.

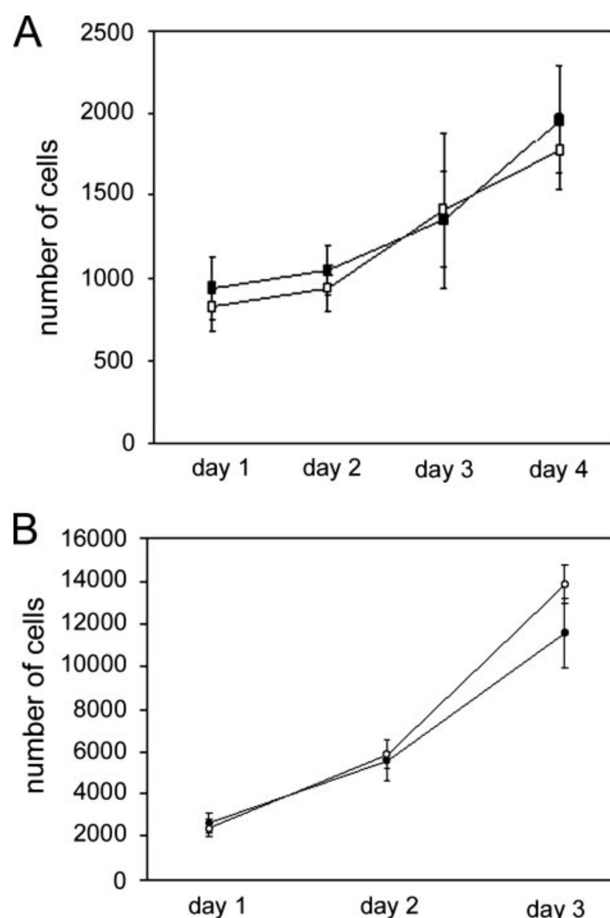


FIGURE 2. **CLP expression does not change the proliferation rate of HeLa cells.** *A*, cell proliferation assays were performed as described under "Experimental Procedures" on stable HeLa cell lines lacking CLP (clone 20, *open squares*) or constitutively expressing CLP (clone 12, *filled squares*). *B*, cell proliferation assays performed on inducible HeLa clone TO-12.2 grown in the absence (*open circles*, lacking CLP) or presence (*filled circles*, expressing CLP) of doxycycline. Results are shown ± S.D. of cell numbers *versus* days in culture.

*CLP Expression Alters the Morphology of Soft Agar Colonies, but Not the Colony-forming Potential of HeLa Cells*—Although CLP appears to have little effect on the proliferation rate of HeLa cells, it may affect their anchorage-independent growth capacity. As shown in Fig. 3*A*, cells constitutively expressing CLP (clone 12) or induced to express CLP (clone TO-12.2 + doxycycline) formed comparable numbers of soft agar colonies as cells lacking CLP. Furthermore, the colony sizes were similar (Fig. 3*B*). However, we noticed a distinct difference in the morphology of colonies formed by cells that express CLP (Fig. 3*C*). CLP-expressing colonies had irregular, diffuse-looking boundaries with numerous individual cells seeming to stray away from the colony edge. By contrast, colonies formed in the absence of CLP expression were more smooth-edged and regular.

*CLP Enhances Wound Healing by Promoting Increased Cell Migration*—The colony-forming assay indicated that CLP expression might affect cell migration. Therefore, a wound healing assay was used as a standard test to compare cell migration in CLP-expressing and control cells. Fig. 4*A* shows a representative example with cells from HeLa clone TO-12.2. Cells induced with doxycycline migrated into the wound significantly faster than cells without CLP induction. Western blot-

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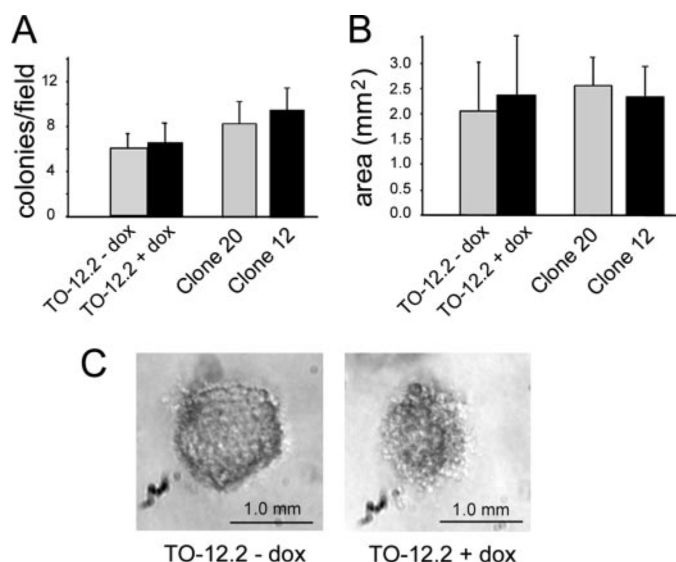


FIGURE 3. **CLP expression alters the morphology, but not the number and size, of soft agar colonies formed by HeLa cells.** Soft agar colony-forming assays were performed as described under "Experimental Procedures." *A*, number of colonies/microscope field + S.D. for cells of clone TO-12.2 grown in the absence (*gray bars*) or presence (*black bars*) of doxycycline, and of clone 20 (lacking CLP) and clone 12 (expressing CLP). *B*, colony size (area in $mm^2$) + S.D. for cells grown from the clones shown in *panel A*. *C*, micrographs of representative colonies from clone TO-12.2 grown in the absence (− *dox*) or presence (+ *dox*) of doxycyline. Note the more ragged morphology of the colony formed from cells expressing CLP (*right panel*).

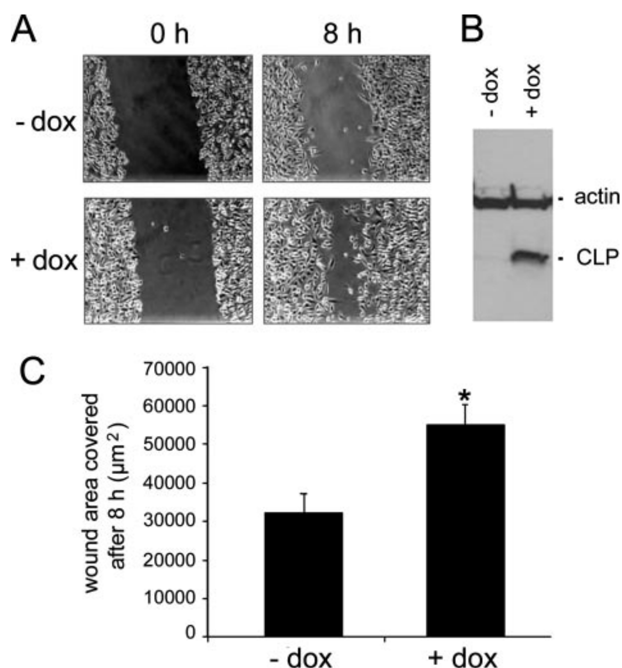


FIGURE 4. **CLP expression increases migration in HeLa cells.** Wound healing scratch assays were performed with CLP-inducible HeLa clone TO-12.2 grown in the absence (− *dox*) or presence (+ *dox*) of doxycycline. *A*, representative photomicrographs of cells around the wound edge at 0 and 8 h after wounding. *B*, these same cells were then lysed and aliquots subjected to SDS-PAGE and Western blotting to confirm the induction of CLP expression. Western blotting for $\beta$-actin was done to control for protein loading. *C*, quantitative analysis of the wound healing capacity of clone TO-12.2 cells without (− *dox*) or with (+ *dox*) induction of CLP expression. The wound area covered after 8 h of incubation was determined as described under "Experimental Procedures" from six independent experiments and is shown with the S.D. *, difference significant at $p \leq 0.05$.

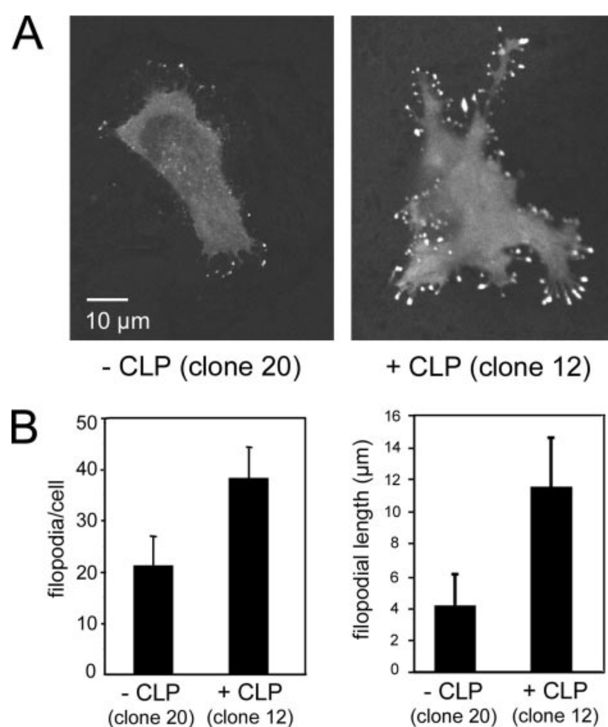


FIGURE 5. **CLP expression increases the number and length of filopodia in HeLa cells.** *A*, representative fluorescence photomicrographs of cells from clone 20 (lacking CLP) and clone 12 (expressing CLP) transfected with EGFP-Myo10 to illustrate the differences in filopodial length and number. *B*, bar graphs (+ S.D.) of the number of filopodia/cell (*left panel*) and of the average filopodial length (*right panel*) in cells lacking CLP (*clone 20*) or expressing CLP (*clone 12*).

ting confirmed that the doxycycline-induced cells indeed expressed CLP (Fig. 4*B*). Quantitative measurements of the wound area remaining open after 8 h showed that CLP-expressing cells had covered an area ~1.7 times greater than cells that did not express CLP (Fig. 4*C*). The improved wound healing capacity of CLP-expressing cells appears to be due to their increased motility and directional migration into the open space. Within minutes of wounding, CLP-expressing cells at the wound edge assumed an elongated shape and extended protrusions toward the wound. By contrast, control cells were oriented more randomly and contained fewer directional protrusions toward the wound (not shown).

*CLP Expression Increases the Number and Length of Myosin-10 Containing Filopodia*—Unconventional myosin-10 is a specific target protein of CLP (7). Myo-10 is involved in filopodia extension and intrafilopodial motility (13–15). We reasoned that the increased motility of CLP-expressing HeLa cells in the wound healing assay might be mediated by Myo-10. To visualize Myo-10 containing filopodia, we transfected HeLa cells from clone 20 (lacking CLP) and clone 12 (expressing high levels of CLP) with EGFP-Myo10. As illustrated in Fig. 5*A*, CLP-expressing cells displayed a striking increase in the number of Myo-10-positive filopodia compared with control cells lacking CLP. In addition, the average length of the filopodia was significantly greater in CLP-expressing cells. A quantitative evaluation of these observations is shown in Fig. 5*B*. Taken together, the results suggest that CLP acts on Myo-10 to induce more and longer filopodia in the cell.

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*CLP Increases Filopodia-dependent Cell Motility by Stabilizing Myosin-10 Expression*—In addition to the "starburst" pattern of EGFP-Myo10-labeled filopodia in the CLP-expressing cells, we observed that the overall level of Myo-10 fluorescence was consistently higher in these cells than in the control cells lacking CLP. This was unexpected because identical transfection conditions were used for all cells. To examine this further, we used an affinity-purified antibody against Myo-10 (MX-117, a gift from Dr. Richard Cheney) to determine the level of endogenous myosin-10 in control (clone 20) or CLP-expressing cells (clone 12). The results (Fig. 6*A*) show a striking increase in *endogenous* Myo-10 in CLP-expressing cells. We corroborated these data by Western blotting for endogenous Myo-10. Fig. 6*B* shows that endogenous Myo-10 was rapidly up-regulated following CLP induction in stably transfected HeLa cells. Within 2 h of induction, the amount of Myo-10 was significantly increased from basal levels and remained elevated for the duration of the experiment. To show that this is not due to CLP-mediated transcriptional up-regulation, but rather to stabilization of the Myo-10 protein, we transfected regular HeLa cells with recombinant EGFP-Myo10-(1–980) either alone or together with CLP. EGFP-Myo10-(1–980) encodes a GFP-tagged truncated Myo-10 fragment consisting of amino acids 1–980 that contains the CLP-binding "neck" domain but lacks the C-terminal half of the molecule. Fig. 6*C* shows that cells co-transfected with CLP contained significantly higher levels of EGFP-Myo10-(1–980) than cells transfected with EGFP-Myo10-(1–980) alone. Finally, we performed semiquantitative reverse transcription PCR with Myo10-specific primers on cells that do (clone 12, TO-12.2 + dox) or do not (clone 20, TO-12.2 − dox) express CLP. As shown in Fig. 6*D*, the levels of endogenous Myo10 mRNA were comparable in these cells regardless of their CLP expression status. Taken together, the data thus suggest that CLP up-regulates Myo-10 by increasing its stability, thereby allowing increased Myo-10 function in filopodial dynamics and cell motility.

*Myosin-10 Knock Down Prevents the CLP-mediated Increase in Cell Motility*—To confirm that the effects of CLP overexpression on cell motility are mediated by Myo-10, we transfected CLP-expressing cells with siRNA against Myo-10 or with control siRNA and performed wound healing assays as before. Three separate Myo-10-specific siRNAs were used, yielding similar results. Cells transfected with Myo-10-specific siRNA exhibited a significant decrease in their ability to migrate into the wound when compared with cells transfected with control siRNA (Fig. 7*A*). Importantly, CLP expression remained high in the Myo-10 siRNA-transfected cells whereas Myo-10 expression was strongly suppressed, demonstrating the efficiency and specificity of the siRNA treatment (Fig. 7*B*). The reduced wound healing capacity of human Myo-10 siRNA-treated cells was partially rescued upon transfection with a GFP-tagged bovine Myo-10 construct (a kind gift from Dr. Richard Cheney); however, because of the limited transfection efficiency of GFP-Myo-10, the phenotype was only partially restored (data not shown). Taken together, these results indicate that the effects of CLP on cell motility are dependent on Myo-10 function and directly confirm the functional link between these two proteins.

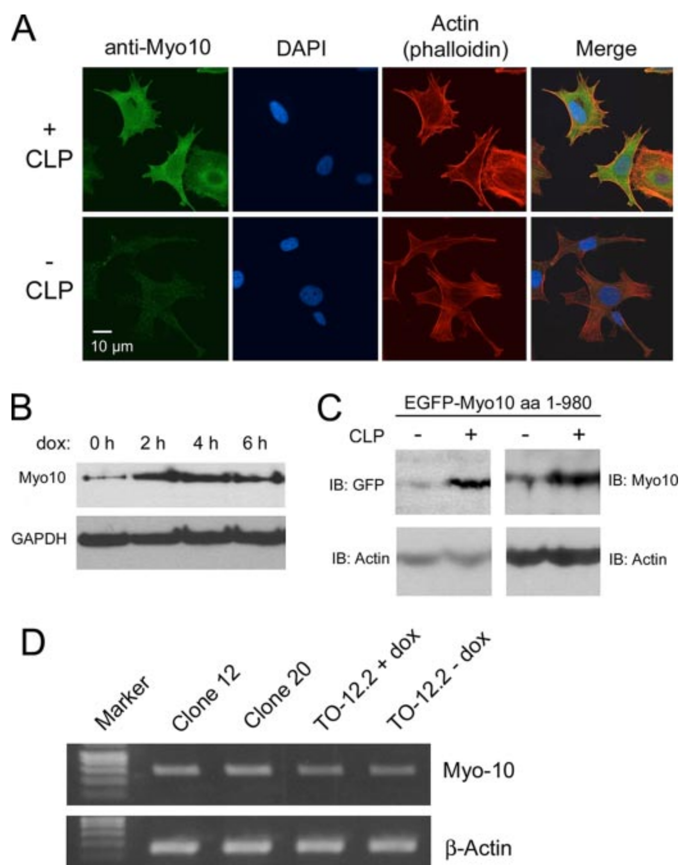


FIGURE 6. **CLP up-regulates endogenous and exogenously expressed Myo-10 at the protein level.** *A*, immunofluorescence of HeLa cells expressing CLP (*top panels*) or lacking CLP (*bottom panels*) stained for endogenous Myo-10 using anti-Myo10 antibody MX-117 and Alexa-fluor 488 secondary antibody (*green*). Cells were co-stained for actin using rhodamine-phalloidin (*red*); nuclei were stained with 4′,6-diamidino-2-phenylindole (*blue*). Merged pictures are shown on the *right*. Note the significant increase in Myo-10 staining (green fluorescence) in cells expressing CLP compared with cells lacking CLP. *B*, Western blot demonstrating up-regulation of endogenous Myo-10 in TO-12.2 HeLa cells as a function of time after CLP induction with doxycycline. Aliquots of cells exposed to doxyxycline for the time indicated on *top of each lane* were used for Western blotting and probed for Myo-10 (*top panel*) and GAPDH (*bottom panel*, loading control). *C*, Western blots showing up-regulation of exogenously expressed EGFP-Myo10-(1–980) in cells expressing CLP. HeLa cells were transfected with a plasmid encoding EGFP-tagged Myo10-(1–980) alone or together with a plasmid encoding CLP as indicated on *top of each lane*. 24 h later, the cells were lysed and aliquots used for immunoblotting (*IB*) to detect the recombinant Myo-10 either via its GFP tag (*top left panel*) or by using an antibody against Myo-10 (*top right panel*). The blots were also probed for actin as loading control (*bottom panels*). *D*, semiquantitative reverse transcription PCR to determine endogenous Myo-10 mRNA was performed as described under "Experimental Procedures" on cells that do (*clones 12* and *TO-12.2 + dox*) or do not express CLP (*clones 20* and *TO-12.2 − dox*). Aliquots of the products obtained after 25 cycles (linear phase of amplification) were run on an agarose gel and compared with an internal control (*β-actin*). DNA size markers are shown in the *left lane*; the Myo-10-specific product is 573 bp and the β-actin fragment 353 bp.

## DISCUSSION

Calmodulin-like protein was first identified as a protein down-regulated in breast cancer and absent or strongly reduced in all transformed cell lines tested (2, 3, 8). However, whether the loss of CLP expression plays a causal role in tumorigenesis is unknown. The results of this study show that stably transfected HeLa cells proliferate normally in the presence of CLP, suggesting that CLP down-regulation is not a prerequisite for tumor cell growth. Why then is down-regulation of CLP

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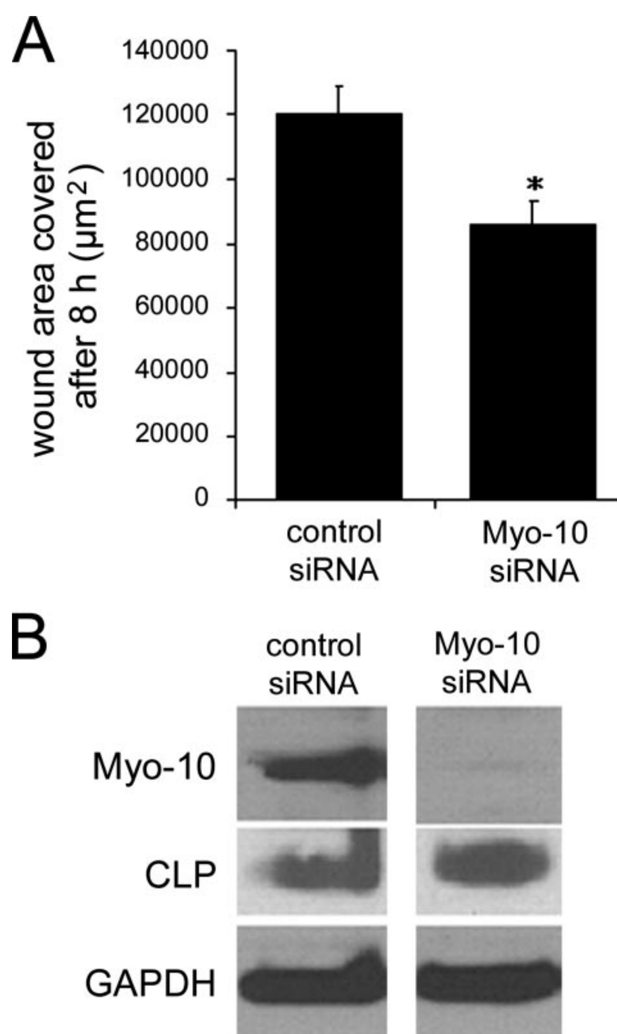


FIGURE 7. **Down-regulation of Myo-10 by siRNA decreases the wound healing capacity in CLP-expressing cells.** *A*, quantitative analysis of the wound healing capacity of CLP-expressing cells from clone 12 transfected with control siRNA or with specific siRNA against Myo-10. Wound healing assays were performed 24 h after siRNA transfection as described under "Experimental Procedures." *, difference significant at $p \leq 0.05$. *B*, cells treated as in *panel A* were immediately lysed following the 8-h wound healing assay and subjected to SDS-PAGE and Western blotting for Myo-10, CLP, and GAPDH as a loading control.

expression such a consistent finding in tumorigenesis? In normal tissues, CLP expression appears to be restricted to stratified epithelia such as those from the skin, breast, and prostate (3, 9). CLP expression appears to be linked to (terminal) differentiation of skin epithelial cells, with CLP levels being up-regulated in suprabasal cells as they migrate toward the upper epidermal layer (9). The loss of CLP expression in epithelial cancers thus likely reflects the transition of tumor cells from a normally differentiating to a poorly differentiated phenotype.

What is the function of CLP during epithelial differentiation? Previous work has shown that CLP is a specific light chain of the unconventional myosin-10 (7). Myo-10 has been implicated in a variety of cell processes including filopodial extension, cell adhesion, and phagocytic cup formation (16, 17). This multifunctional molecular motor binds and transports β-integrins and Mena/VASP proteins (14, 15) and may act as a molecular link between microtubules and the actin cytoskeleton (18). Myo-10 is thought to function in directional cell migration by moving cargo, including Mena/VASP and integrins, to the leading edge of the cell and into filopodial tips. Mena/VASP and integrins are required for the regulation of actin polymerization and the establishment of new adhesive structures, respectively. These features are required in epithelial cells as they migrate toward the outer layers and form new adhesive contacts during terminal differentiation.

Overexpression of Myo-10 leads to the formation of new and elongated filopodia in several cell types (13, 15). Our study demonstrates that the same phenotype results from expression of CLP. HeLa cells expressing CLP showed more and longer filopodia and migrated faster into an open wound. siRNA-mediated down-regulation of Myo-10 prevented the increase in cell motility even in the presence of high levels of CLP and thus directly confirmed the functional connection between CLP and Myo-10. By increasing the stability of Myo-10, CLP up-regulates endogenous Myo-10 and thereby enhances its function. This constitutes a novel role for CLP as a myosin light chain. The (calmodulin-like) light chains of unconventional myosins are thought to provide stiffness to the myosin lever arm and thereby regulate the step size of myosin on the actin filament (19–21). CLP binds to the IQ3 motif in Myo-10 (7) where it may fulfill a dual function stabilizing the lever arm of the motor and protecting the protein against degradation. No structural information is currently available on Myo-10, but PEST (proline, glutamate, serine, and threonine-rich) sequences located ~170 amino acids C-terminal to the IQ motifs are probable cleavage sites for calpain or other proteases (10). In the presence of CLP, the PEST sites may be conformationally masked, protecting full-length Myo-10 against proteolysis. Further studies using purified Myo-10 will be needed to address this issue.

As an EF-hand protein, CLP is a possible sensor of elevated $Ca^{2+}$ during epithelial differentiation. In skin, as the layers of keratinized epithelial cells get pushed further toward the surface $Ca^{2+}$ levels increase partly due to dehydration. Myo-10 contains three IQ motifs that can all be occupied by calmodulin light chains. *In vitro*, it has been reported that elevated $Ca^{2+}$ levels (>1 $\mu$M) cause one CaM molecule to dissociate from Myo-10, resulting in loss of motor function (22). However, under these conditions CLP will remain bound to Myo-10 as its binding to the IQ3 motif requires $Ca^{2+}$ (7). Thus, CLP could extend the functional life of Myo-10 in the high $Ca^{2+}$ environment prevailing during terminal keratinocyte differentiation. In agreement with this hypothesis, CLP expression increases in the suprabasal, spinous, supraspinous, and granulated layers of keratinocytes in a pattern that closely follows terminal differentiation. During this time cells migrate from the basal layer and form cell-cell contacts that tightly connect cells and become an essential barrier to the external environment. Appropriate temporal induction of CLP may be necessary to promote the migration and/or changes in adhesion of cells through stabilization of Myo-10. Future studies will be needed to determine whether CLP expression, via its effect on Myo-10 function, is essential for normal (skin) epithelial development *in vivo*.

In conclusion, we have shown that CLP expression does not inhibit cell proliferation or anchorage-independent growth of

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HeLa cells but results in phenotypic changes consistent with overexpression of Myo-10. Mechanistically, CLP performs its function through stabilization of Myo-10. Myo-10 is highly unstable, as is typical for a protein involved in dynamic cell processes and signaling. By increasing its stability, CLP assumes a major role in regulating Myo-10 function.

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