Calponin is an extensively studied actin-binding protein, but its function is not well understood. Among three isoforms of calponin, h2-calponin is found in both smooth muscle and non-muscle cells. The present study demonstrates that epidermal keratinocytes and fibroblast cells express significant amounts of h2-calponin. The expression of h2-calponin is cell anchorage-dependent. The levels of h2-calponin decrease when cells are rounded up and remain low when cells are prevented from adherence to a culture dish. h2-calponin expression resumes after the floating cells are allowed to form a monolayer in plastic dish. Cell cultures on polyacrylamide gels of different stiffness demonstrated that h2-calponin expression is affected by the mechanical properties of the culture matrix. When cells are cultured on soft gel that applies less traction force to the cell and, therefore, lower mechanical tension in the cytoskeleton, the level of h2-calponin is significantly lower than that in cells cultured on hard gel or rigid plastic dish. Force-expression of h2-calponin enhanced the resistance of the actin filaments to cytochalasin B treatment. Keratinocyte differentiation is accompanied by a mechanical tension-related up-regulation of h2-calponin. Lowering the tension of actin cytoskeleton by inhibiting non-muscle myosin II ATPase decreased h2-calponin expression. In contrast to the mechanical tension regulation of endogenous h2-calponin, the expression of h2-calponin using a cytomegalovirus (CMV)2 promotor was independent of the stiffness of culture matrix. The results suggest that h2-calponin represents a novel manifestation of mechanical tension responsive gene regulation that may modify cytoskeleton function.
MATERIALS AND METHODS

Specific Antibodies—A rabbit polyclonal antiserum (RAH2) raised against mouse h2-calponin with a weak cross-reaction to h1-calponin has been described previously (36). Monoclonal antibody (mAb) 1D2 against human h2-calponin was developed as described previously for the production of anti-mouse h2-calponin mAbs (13). Briefly, human h2-calponin cDNA was cloned using reverse transcription-coupled polymerase chain reaction as described previously (36) and expressed in Escherichia coli for protein preparation (37). Purified human h2-calponin was used to immunize 8-week-old female Balb/c mice in a short-term immunization protocol (38). Spleen cells were harvested from the immunized mouse for fusion with SP2/0-Ag14 mouse myeloma cells. Hybridoma colonies were screened by indirect enzyme-linked immunosorbent assay and subcloned three times to establish a stable cell line. The mAbs were produced in the forms of hybridoma culture supernatant and mouse ascites fluids. Enzyme-linked immunosorbant assay immunoglobulin isotyping (Invitrogen) determined its subclass as IgG2b, κ.

Western Blotting—PAGE and Western blotting were carried out as described previously (38) to examine the expression of calponin in mouse skin tissues and human keratinocyte and fibroblast cultures. The samples were homogenized in SDS gel electrophoresis sample buffer containing 2% SDS and analyzed on 12% gel with an acrylamide:bisacrylamide ratio of 29:1 in the Laemmli buffer system. After electrophoresis, the gels were fixed and stained with Cooamassie Blue R250 to confirm sample integrity and protein contents. Protein bands in unfixed duplicate gels were transferred to nitrocellulose membrane for Western blotting with the anti-calponin, anti-tropomyosin, or anti-involucrin antibodies. The specific protein bands recognized by the first antibodies were revealed by using alkaline phosphatase-labeled anti-rabbit IgG or anti-mouse IgG second antibody (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chromogenic substrate reaction. Purified h2- and h1-calponins (37) was used as control.

Immunohistochemistry—To examine the in vivo expression of h2-calponin in epidermal keratinocytes, paraffin sections of epidermal scar (anonymously provided by the Skin Disease Research Center at Case Western Reserve University School of Medicine) were stained with anti-h2-calponin mAb 1D2 that had no cross-reaction to h1-calponin followed by horseradish peroxidase-labeled anti-mouse IgG second antibody (Sigma) and 0.05% H2O2/3,3′-diaminobenzidine substrate reaction using standard immunohistochemical methods (46). To avoid background staining of tissue sections by immunosera and mouse ascites fluid (data not shown), 1D2 hybridoma culture supernatant was used. The morphology of tissue sections was outlined by counterstaining of the slides with 0.6% hematoxylin for 20 s.

Cell Cultures—Primary keratinocytes were isolated from human foreskin samples (47) and cultured in serum-free keratinocyte media (Invitrogen) with supplements (50 μg/ml bovine pituitary extract, 5 ng/ml human recombinant epidermal growth factor; Invitrogen) at 37 °C in 5% CO2. Culture media were replaced every 3 days. The monolayer cells around 70% confluence were passed at 1:5 ratio, and cells at second or third passages were used for experiments. The methods for culturing human epidermal keratinocytes were described in detail previously (48, 49).

Human Fibroblast Cell Line KD (American Type Culture Collection, CRL 1295) was cultured in Dulbecco’s-modified Eagle medium containing 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (50 IU/ml) at 37 °C in 5% CO2.

SM3 is an immortalized cell line derived from rabbit aortic smooth muscle (50) and has ceased endogenous calponin expression (13). Stable transfected SM3 cells force-expressing h2-calponin driven by the CMV promoter in pcDNA3.1 vector (13) were used in the present study to investigate h2-calponin regulation and functional effect. The sense cDNA construct expresses non-fusion full-length mouse h2-calponin protein for authentic functional characterization, and antisense cDNA-transfected cell lines were used as negative control. The transfected SM3 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (50 IU/ml), and G418 (100 μg/ml) at 37 °C in 5% CO2.

Immunofluorescence Microscopy—Precleaned glass coverslips were coated with 0.1% gelatin and dried under UV radiation for 3 h before being placed in the culture dish. Keratinocytes and KD cells were seeded to grow a monolayer on the coverslips. After 3 days of culture, the cells grown on coverslips were washed with Dulbecco’s phosphate-buffered saline (D-PBS; 136.8 mM NaCl, 2.68 mM KCl, 8.1 mM NaHPO4, 1.47 mM KH2PO4, pH 7.2). The cells were then fixed with cold acetone for 20 min. Immunofluorescence microscopy was carried out as described previously (13) to examine the cellular localization of h2-calponin and tropomyosin. Immunofluorescence microscopy was used to distinguish the rabbit and mouse first antibody staining representing the localization of calponin and tropomyosin, respectively, using a Zeiss Axiosvert 100H phase contrast-epifluorescence microscope with two sets of filters (CZ915 and CZ909). Actin stress fibers in the cell were examined by rhodamine-labeled phalloidin (Sigma) staining. The localization of h2-calponin and tropomyosin in KD cells were also examined by confocal microscopy at the Neurobiology Core Facility at Case Western Reserve University School of Medicine.

Examination of Cell Plating Time Dependence of h2-calponin Expression—To investigate the expression of h2-calponin during the time course of monolayer cell culture, preconfluent keratinocytes were trypsinized and seeded at high and low densities (initial density = 9 × 104 cells/60-mm dish, respectively). The cells were suspended from the culture dishes after 1, 2, 3, 4, and 5 days using the Versene solution (0.037 mM EDTA, 136.4 mM NaCl, 2.68 mM KCl, 8.1 mM NaHPO4, pH 7.2) and washed 3 times with D-PBS. Omitting trypsin digestion during the cell harvest avoids artificial degradation of cellular proteins. SDS gel sample buffer containing 2% SDS was used to lyse the cells, and total cellular protein was extracted by vortexing. Levels of h2-calponin in high and low density keratinocyte cultures at the post-plating time points were examined by Western blot analysis using anti-h2-calponin antibody RAH2. KD fibroblasts and stable transfected SM3 cells were examined similarly for the plating time effect on the levels of h2-calponin.

Examination of Matrix Adhesion-dependent Expression of h2-calponin—To examine the effects of matrix attachment on the expression of h2-calponin in cultured cells, continuous vibration was applied to keratinocytes, KD fibroblasts, and SM3 cells to prevent cell attachment to tissue culture dishes (51). Incubated on a humidified incubator at 37 °C, the cultural dishes were placed on an orbital shaker (Bel-Art Products, Pequannock, NJ) driven by a magnetic stirrer at 80 rpm. The cells were harvested after 3 days of culture in vibration to examine the levels of h2-calponin. After washed with PBS, the cells were lysed in SDS-gel sample buffer and examined by Western blotting anal-
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ysis as described above. The viability of the floating cell aggregates was confirmed by growing into monolayers after re-seeding on tissue culture dishes and incubated without vibration. The expression of h2-calponin in the cells after re-seeding was examined as described above.

Examination of Culture Matrix Stiffness-dependent Expression of h2-calponin—To compare with the rigid plastic cultural dish, a thin layer of polyacrylamide gel was prepared on glass coverslips to provide a soft matrix for cell culture as described previously (30, 34, 52). The coverslips were passed briefly through the inner flame of a Bunsen burner. A drop of 0.1 N NaOH was smeared across the surface of the coverslip and air-dried. Thereafter, the coverslips were treated with 3-aminopropyltrimethoxysilane and then with 0.5% glutaraldehyde. Polyacrylamide gels of different stiffness (very hard, 10% gel with an acrylamide:bisacrylamide ratio of 40:1; hard, 3% gel with an acrylamide: bisacrylamide ratio of 14:1; soft, 3% gel with an acrylamide:bisacrylamide ratio of 74:1) were polymerized in between the treated coverslip at the bottom and an untreated coverslip on the top. After polymerization, the untreated coverslip was removed to expose the ~100-μm thin layer of gel to be used as cell culture matrix. Previous studies have shown that the stiffness of the polyacrylamide gel matrix is directly proportional to the concentration of bisacrylamide cross-linker (30, 34, 52).

After treating the gel with a bifunctional cross-linker (1 mm sulfo-SANPAH, Pierce) in 50 mM HEPES, pH 8.5 (30), Type I collagen was applied to the gel surface followed by gently shaking overnight at 4 °C for cross-linking to the gel. Gels were then washed with D-PBS and sterilized with UV irradiation for 3 h. The gel was soaked in culture media at 37 °C for 1 h before plating cells.

To examine the effect of the tension that the culture matrix applied to the cell through traction force on the expression of h2-calponin, keratinocytes were seeded on a plastic culture dish or polyacrylamide gels of different stiffness. The cells were harvested after 3 days of culture by directly lysing in SDS gel sample buffer after D-PBS washes. The level of h2-calponin was examined by Western blot analysis with the anti-h2-calponin antibody RAH2 as described.

Measurement of Cell Spreading Area—Monolayer keratinocytes cultured on plastic dish or polyacrylamide gels of different stiffness were photographed at 4, 12, 24, 48, and 72 h of culture. The NIH Image program Version 1.61 was used to measure the two-dimensional spreading area of randomly selected non-overlapping cells.

Ca2+ Induction of Keratinoctye Differentiation—To examine the expression of h2-calponin during keratinocyte differentiation in vitro, cells were plated on plastic culture dishes or soft polyacrylamide gel (3% with an acrylamide:bisacrylamide ratio of 74:1). One day after plating, the concentration of CaCl2 in culture media was increased to 0.3 mM. The cells were harvested after 48 h in the high Ca2+ media for Western blotting examination of h2-calponin as described above. The expression of involucrin, an established cell differentiation marker, in epidermal keratinocytes was examined by Western blot to monitor the induction of differentiation (53–55).

Cytochalasin B Treatment—To investigate the effect of h2-calponin on actin cytoskeleton, stable transfected SM3 cells expressing h2-calponin were plated on gelatin-coated coverslips. After 3 days of culture, the cells were treated with 0.5, 0.75, and 1.0 μg/ml cytochalasin B (Sigma) at 37 °C for 30 min. h2-calponin-negative SM3 cells were examined as the control. The cytochalasin B stock was prepared in Me2SO, so a 0.2% Me2SO-treated control group of cells was also examined. The cells were washed with PBS and fixed with cold acetone. The structure of stress fibers stained with rhodamine-labeled phalloidin was examined as above and evaluated for the resistance to cytochalasin B treatment.

Blebbistatin Treatment—To examine the effect of reducing cytoskeletal tension, which is mainly built up by myosin II motor activity on the expression of h2-calponin in cells, NIH 3T3 cells were cultured on plastic dish and gelatin-coated coverslips (3 × 104 cells/35-mm dish). After 3 days of monolayer culture, the cells were treated with 100 μM blebbistatin (Sigma), a myosin II ATPase inhibitor (56), for 3 days. Parallel cultures in normal media or containing 0.2% MeSO (solvent for making the blebbistatin stock) were examined as controls. The cells in culture dish were harvested by lysis in SDS-PAGE sample buffer after PBS washes, and the levels of h2-calponin were determined by Western blot analysis as above. The cells on the coverslips were fixed with cold acetone, and the actin stress fibers were stained with rhodamine-conjugated phalloidin. The fluorescence and phase contrast images were viewed under a Zeiss Axiosvert 100H fluorescence microscope.

Data Analysis—Densitometry analysis of SDS-gel and Western blots was done on digital images scanned at 600 dots/inch, and the NIH Image program Version 1.61 was used to quantify the levels of h2-calponin and tropomyosin expression. The calponin and tropomyosin bands detected in Western blots were normalized by the amounts of actin in equally loaded SDS gel. The quantitative data of the cell area, h2-calponin, and tropomyosin levels are presented as the mean ± S.D. Statistical analysis was done using the Microsoft Excel computer program.

RESULTS

h2-calponin Expression in Epidermal Keratinocytes—The expression of h2-calponin in mouse and human skin tissues was examined by Western blot and immunohistochemistry. The results in Fig. 1A show significant amounts of h2-calponin in mouse skin. Normalized by actin

FIGURE 1. h2-calponin expression in epidermal keratinocytes. A, total protein extracts from mouse abdominal and footpad skin tissues were analyzed by Western blots using the anti-h2-calponin polyclonal antibody RAH2 and anti-h1-calponin mAb CP1. Purified mouse h1- and h2-calponin proteins were used as the control. Normalized by the level of actin, the blots show a significant level of h2- but not h1-calponin in the mouse skin, especially the footpad. B, thin paraffin sections of human epidermal scar tissues were examined by immunocytochemistry with anti-h2-calponin mAb 1D2 and SP2/0 myeloma cultural supernatant control. The results show h2-calponin expression in the keratinocyte layers. C, Western blots using anti-h2-calponin polyclonal antibody RAH2 and anti-h1-calponin mAb CP1 on total protein extracts from human keratinocytes cultured 3 days on a plastic dish detected high levels of h2- but not h1-calponin. The sample loading was normalized by the level of actin, and purified mouse h1- and h2-calponins were included as control.
contents, mouse footpad has more h2-calponin expression than that in the abdominal skin, in agreement with the thickness of keratinocyte layer. No h1-calponin was detected in the mouse skin. Immunohistochemical staining detected h2-calponin expression in the keratinocytes of human skin enriched in the basal layer and the early suprabasal layers (Fig. 1B). Western blots with either polyclonal (RAH2) or monoclonal (1D2) antibody confirmed the high level expression of h2-calponin in primary cultures of human epidermal keratinocytes (Fig. 1C). h1-calponin was not detected.

Expression of h2-calponin Is Dependent on the Time of Culture and Independent of Cell Density—Human epidermal keratinocytes from 3-day-old preconfluence cultures, human epidermal keratinocytes were passed on plastic dishes at low or high densities. The cells were harvested at 1, 2, 3, 4, and 5 days after plating, and the total protein extracts were examined by SDS-PAGE and Western blot using anti-h2-calponin polyclonal antibody RAH2 (A). Normalized against the actin band in the accompanying SDS-gel, densitometry quantification of the Western blots was used to compare the levels of h2-calponin expression (B). The results show that independent of the high (solid columns) or low (open columns) cell densities (the cells were at 100 and 60% confluence at 3 days in culture, respectively), the expression of h2-calponin was low at 1 day after plating (p < 0.001) and returned to the maximum level 3 days after plating. The results are summarized from six individual experiments.

Expression of h2-calponin Is Dependent on Cell Anchorage to the Culture Dish—Two factors may confer the recover of h2-calponin expression during the 3 days of culture after cell passage. One is the culture time after passage, and the other is the re-attachment to the culture dish. We investigated which factor determines h2-calponin expression by producing floating cell cultures. The results in Fig. 3, A and B, show that when keratinocytes were prevented from attachment to the culture dish by continuous vibration, the cells grew in multi-cell aggregates with significantly decreased h2-calponin expression.

Because primary human epidermal keratinocytes may not survive well in the floating culture, the observed phenotype after passages often vary between experiments. We, therefore, verified this observation using the human fibroblast cell line KD. The Western blots in Fig. 3, C and D, show a significant expression of h2-calponin in the KD fibroblast monolayer cultured on plastic dishes. When KD cells were cultured with continuous vibration, they also grow in multi-cell aggregates (Fig. 3C). Western blot analysis showed that the floating KD cells completely lost h2-calponin, in sharp contrast to the high level expression in monolayer cells attached on plastic dish (Fig. 3D). After the floating cells were re-plated and cultured without vibration, they attached to the plastic dishes and regained high level expression of h2-calponin (Fig. 3D). The results verify the viability of the floating cells cultured under vibration and further demonstrate...
that h2-calponin expression is dependent on the anchorage to the plastic culture dish other than the culture time after passage. Consistent with the independence of cell-cell contacts as shown in the cell density comparisons (Fig. 2), the aggregation of floating cells does not induce h2-calponin expression.

Matrix Stiffness Determines h2 Calponin Expression—A plastic dish provides a rigid culture matrix that allows the attached cell to exert a high traction force, applying a high mechanical tension to the cellular structure. To investigate a hypothesis that the high mechanical tension in monolayer cells growing on a plastic tissue culture dish induces h2-calponin expression, we used polyacrylamide gel to provide cell culture matrices of different stiffness. It has been documented that the different stiffness of the gel matrix will produce different strength of traction forces to the cell (29). The results in Fig. 4 show that hard gel matrices produced high level of h2-calponin expression similar to that in cells cultured on plastic dishes, indicating that the difference in matrix material (collagen-coated polyacrylamide gel or plastic) has little effect on h2-calponin expression. On the other hand, cells cultured on the soft gel had significantly lower levels of h2-calponin expression in comparison to that of the hard gel or plastic controls ($p < 0.001$). The results support a novel observation that mechanical tension determines the expression of h2-calponin.

It has been established that centripetal-directed traction forces exerted on cells and cell areas are increased with increasing stiffness of culture matrix (29). The spreading area of keratinocytes at 4 h after plating was smaller when cultured on soft gel than that of the very hard gel or plastic controls (Fig. 5, A and B). The less spreading morphology of the cells cultured on a soft matrix implies that less tension is applied to the cytoskeleton than that in the more spreading cells cultured on hard matrices. Nonetheless, the cell area increases at similar rates during culture under these conditions (Fig. 5C), indicating adjusted cytoskeleton activity.

![Figure 3. Matrix anchorage-dependent expression of h2-calponin in keratinocytes and fibroblasts.](image)

Human epidermal keratinocytes were cultured on plastic cultural dishes steadily as a monolayer or with continuous vibration that prevented the cells from anchoring on the dish. After 3 days of culture, the cells were photographed for phase contrast images (A) and harvested to examine the total protein extracts by Western blotting with anti-h2-calponin polyclonal antibody RAH2 (B). Normalized by the actin level, the results show that the floating keratinocytes growing in multi-cell aggregates had significantly reduced h2-calponin expression. Similarly, phase contrast images (C) show that continuous vibration prevented KD human fibroblasts from anchoring to the cultural dish and produced multi-cell aggregates. When the floating cell aggregates were re-seeded on the plastic dish and cultured without vibration, they attached to the dish to form a monolayer similar to the steady culture control of the same age. The result verifies the viability of the cells in the floating aggregates. D, total protein extracts from the KD cells were examined by SDS-PAGE and Western blotting using anti-h2-calponin antibody RAH2. Normalized by the level of actin, the results show that KD cells lost h2-calponin expression when growing as floating aggregates. The expression of h2-calponin resumed when the floating cells anchored to the cultural dish.
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h2-calponin Increases Actin Filament Resistance to Cytochalasin B—By binding to the barbed ends of actin filament and, thus, inhibiting polymerization, cytochalasins disrupt actin cytoskeleton and can be used to evaluate the stability of actin filaments (58). We investigated the role of h2-calponin in the function of actin cytoskeleton by examining the effects of cytochalasin B on actin stress fibers. SM3 cell provides an endogenous h2-calponin null background to investigate the functional effect of transfective expression of h2-calponin. Normalized against actin, the h2-calponin contents in stable transfected SM3 lines, keratinocytes, and mouse and human fibroblast cell lines showed a wide range of variance (up to ~2.4-fold differences, data not shown) where the levels of h2-calponin expressed in the transfected SM3 cells were within this natural range. The rhodamine-labeled phalloidin stain in Fig. 8A shows that the actin stress fibers in h2-expressing SM3 cells tolerated higher concentrations (>0.75 μM/gel) of cytochalasin B than that of the calponin null control SM3 cells. Tropomyosin has been demonstrated to contribute to the stability of actin filament in cytoskeleton assays (59). Four isoforms of tropomyosin are detected in SM3 cells (hTM2, hTM3, hTM4, and hTM5), and all of them were increased in the presence of forced expression of h2-calponin (Fig. 8, B and C). Moreover, the increases in tropomyosin parallel the force-expressed levels of h2-calponin (r^2 = 0.888, p < 0.05). These results suggest that h2-calponin may regulate the dynamics and function of actin filaments together with the function of tropomyosin. It is worth noting that the tropomyosin contents in non-transfected SM3 cells, keratinocytes, and KD and 3T3 fibroblast cell lines vary significantly (up to ~3-fold differences; data not shown). Therefore, it is not simply the level of Tm that confers the functional effect of h2-calponin. The contribution of Tm to the cell phenotype changes corresponding the presence or absence of h2-calponin remains to be investigated.

Inhibition of Myosin II Results in Decreases in h2-calponin—Blebbistatin is a small molecule inhibitor that selectively binds non-muscle myosin II with high affinity, slows down its ATPase rate, and blocks myosin II in an actin-detached state (60). Blebbistatin provides a tool to reduce mechanical tension built into the actin cytoskeleton (61). When 3-day-old monolayer cultures of NIH 3T3 fibroblasts that express significant levels of h2-calponin (Fig. 9A) were treated with 100 μM blebbistatin, the morphology of the cells started to change within 1 h and remained at a “slack” state (Fig. 9B). h2-calponin expression was determined by Western blot analysis with anti-h2-calponin antibody RAH2. Densitometry quantification results show a significantly lower level of h2-calponin in blebbistatin-treated cells in comparison with that of non-treated (p < 0.01) and 0.2% Me2SO (p < 0.001)-treated cells (Fig. 9C). h2-calponin expression in Me2SO-treated cells was slightly increased (p < 0.01) in comparison to that in non-treated NIH 3T3 cells, suggesting that Me2SO may induce h2-calponin expression. Nonetheless, the opposite change indicates that the trace amount of Me2SO carried over from the blebbistatin stock is not responsible for the decrease in h2-calponin in the blebbistatin-treated group. The results support the hypothesis that mechanical tension in the actin filaments regulates h2-calponin expression. This mechanism is plausible for calponin function in regulating actin-myosin interaction and worth further investigation.

Viral Promoter-directed Expression of h2-calponin Is Independent of Cell Attachment or Matrix Stiffness—The regulation of h2-calponin by mechanical tension applied to the actin cytoskeleton and the role of h2-calponin in stabilizing actin filaments suggested two possible mechanisms of cellular regulation. One is that the mechanical signals regulate the expression of h2-calponin gene. The other is that a reorganization of...
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cytoskeleton in response to mechanical tension alters the accumulation of h2-calponin. To investigate these potential mechanisms, we examined forced expression of h2-calponin by a viral promoter in SM3 cells, an immortalized cell line derived from rabbit aortic smooth muscle that has lost endogenous calponin expression. With a calponin-positive origin implying a compatible cellular environment, SM3 cell provides a calponin null background for investigating the function of exogenous h2-calponin. CMV promoter produced a high level expression of h2-calponin in monolayer SM3 cultures. The results in Fig. 10A show that in contrast to the post-cell roundup decreases of endogenous calponin, h2-calponin expression remained high even under conditions of cell rounding.

FIGURE 5. Cell spreading areas when cultured on matrices of different stiffness. Keratinocytes were cultured on plastic surface or polyacrylamide gels of different stiffness. A, phase contrast images at 3 days of culture show that the cells attached and grew in monolayers on the plastic and gel matrices. The cell spreading areas 4 h after plating were significantly lower (*, p < 0.001) in the soft gel culture as compared with the very hard gel and plastic controls (B). However, the cell spreading areas under the three matrix stiffness conditions increased at similar rates during culture (C).

FIGURE 6. Increased h2-calponin expression during Ca2+-induced differentiation of keratinocytes grown on soft matrix. Human epidermal keratinocytes were plated on a soft polyacrylamide gel or a plastic dish and induced with 0.3 mM CaCl2 at 40–70% confluence for 48 h. A, phase contrast images show that in contrast to non-induced spreading cell culture, the cells in both soft and rigid matrix cultures migrated together upon Ca2+ induction. B, normalized by the levels of actin, total protein extracted from the cells was analyzed by Western blot to examine the expression of h2-calponin and involucrin. The increase in involucrin level upon Ca2+ induction verified the differentiation of keratinocytes in cultures on soft and rigid matrix. Although the expression of h2-calponin was very low in keratinocytes cultured on soft gel, it is significantly up-regulated upon Ca2+ induction (*, p < 0.001). Different from the soft matrix cultures, the expression of h2-calponin in the cells cultured on rigid matrix was already high and did not further increase during differentiation. Five individual experiments each were performed for the soft and rigid matrix differentiation experiments.
h2-calponin seen in keratinocytes (Fig. 2) and fibroblasts (data not shown), the level of CMV promoter force-expressed h2-calponin in SM3 cells was not decreased but slightly increased at 12 and 24 h after cell passage \((p < 0.01)\).

Similar to keratinocytes and fibroblasts (Fig. 3), SM3 cells grew in aggregates under vibration, and the cell aggregates reattached to the dish to form a monolayer when reseeded in steady culture dish (data not shown). Fig. 10B shows that in contrast to the dramatic decrease in endogenous h2-calponin in floating keratinocytes and fibroblasts (Fig. 3), the level of force-expressed h2-calponin was even higher in the floating cells than that in the attached cells. After re-seeding and cultured without vibration, the transfected SM3 cell aggregates anchored to the plastic dish to form a monolayer, and the level of h2-calponin returned to the initial steady level. The distinct responses of endogenous gene and viral promoter-driven expression of h2-calponin to mechanical tension suggest that mechanical tension regulates h2-calponin gene expression rather than affects cytoskeleton accumulation of the protein.

Consistently, when the transfected SM3 cells were cultured on soft gel matrix, the level of h2-calponin did not decrease (Fig. 10C). Instead, as in the floating culture, there was an increase in h2-calponin level in cultures on the soft gel matrix. These results further support that the mechanical tension-dependent expression of endogenous h2-calponin in keratinocytes and fibroblasts is via gene regulation. On the other hand, the low tension-caused increase in h2-calponin expressed by the presumably mechanical tension-independent CMV promoter may reflect an increased accumulation of calponin protein in the actin filaments due to cytoskeleton reorganization, in agreement with h2-calponin proposed function in cytoskeleton remodeling.

**DISCUSSION**

Abundant h2-calponin in Fibroblast and Epithelial Cells—A previous study using an antibody raised against h1-calponin detected an association of calponin with actin in activated platelets and along stress fibers of both fibroblasts and smooth muscle cells (62). Using h1- and h2-calponin-specific mAbs, we detected only h2-calponin in epidermal keratinocytes and fibroblast (Figs. 1 and 3). Therefore, the previously reported fibroblast calponin might be h2-calponin detected by antibody cross-reactions.

An established activity of h1-calponin is the inhibition of smooth muscle actomyosin ATPase (4–6). Structural conservation suggests that h1, h2, and acidic calponins may function via similar molecular mechanisms to regulate actin-myosin interactions. We previously showed that forced expression of h2-calponin in SM3 cells inhibits cytokinesis and the rate of cell proliferation (13). The association of h2-calponin to the actin filament in keratinocytes and fibroblasts (Fig. 7) is consistent with a role in the regulation of actin-based cell motility and other cellular activities (12). Similar to the fact that smooth muscle can contract normally without h1-calponin (36, 63, 64), not all tissues or cultured cells express h2-calponin. Therefore, h2-calponin seems not to be an essential protein for epithelial cells or fibroblasts to grow and function but may represent a regulatory mechanism that fine-tunes the function of actin cytoskeleton.

Mechanical Tension Regulates h2-calponin Gene Expression—Living cells can respond to both the magnitude and distribution of adhesion force (65). In the present study we demonstrated that the expression of h2-calponin in cell cultures is determined by cell anchorage to the cul-

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**FIGURE 7. Association of h2-calponin with actin cytoskeleton.** Human keratinocytes and KD fibroblasts were cultured on gelatin-coated glass coverslips. A, preconfluent monolayer keratinocyte cultures were examined by immunofluorescence microscopy with the rabbit anti-h2-calponin antibody RAH2 or anti-tropomyosin (hTM4) mAb LC24 in comparison with TRITC-phalloidin stained actin filaments. B, immunofluorescence assay on KD fibroblast culture was carried out using anti-h2-calponin antibody RAH2 and anti-tropomyosin (hTM5) mAb CG3 together with TRITC-phalloidin control. C, confocal microscopy of the double-stained KD cells demonstrates the co-localization of h2-calponin (green) and Tm (red) in actin stress fibers (yellow color). Plotting the intensity of co-localized h2-calponin and Tm stains (right panel) shows a positive correlation.
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The steady tension in actin stress fibers depends on the motor function of non-muscle myosin II. Inhibition of myosin II ATPase by blebbistatin reduces the mechanical tension of actin cytoskeleton (61). The data that blebbistatin treatment resulted in a decreased level of h2-calponin together with diminished stress fibers in NIH 3T3 cells cultured on plastic dish supports the hypothesis that h2-calponin is regulated by actomyosin-based cytoskeletal tension. This novel mechanism is consistent with the potential role of h2-calponin in regulating the function of actin filaments in non-muscle cells similar to the h1-calponin regulatory effect on actin-myosin interaction in smooth muscle.

In contrast to the endogenous h2-calponin in keratinocyte and fibroblasts, no post-passage transient decrease in levels was found for the transfective-expressed h2-calponin in SM3 cells (Fig. 10A). The viral promoter-driven expression of h2-calponin was independent of matrix stiffness (Fig. 10, B and C), indicating a primary control at the level of gene regulation. However, the decrease in h2-calponin levels after cell round-up during passages is a rapid process (data not shown). Proteolytic degradation may play a critical role in this process. The lack of post-round-up decreases of h2-calponin in SM3 cells suggests that the proteolytic mechanism is not a ubiquitous mechanism in all cells, and the mechanisms remain to be investigated. Although keratinocytes and KD fibroblasts reattach to the culture dish within 30 min after re-plating, 3 days are needed for their h2-calponin level to return to the high level before the passage. This slow recovery suggests that rapid degradation may have removed most of the h2-calponin protein, and de novo synthesis in response to high mechanical tension is required to restore h2-calponin levels in the cell. The lack of rapid degradation of exogenous h2-calponin in SM3 cells indicates that the proteolytic regulation may be cell type-specific.

Up-regulation of h2-calponin during Epidermal Keratinocyte Differentiation—Increases in extracellular Ca" concentration result in termination of cell growth and induction of a terminal differentiation
The Role of h2-calponin in the Function of Actin Cytoskeleton—h2-calponin association to the actin filaments and its regulation by the mechanical tension of the cytoskeleton indicate functional significance. A previous study observed that transfective expression of green fluorescent protein fusion h1-calponin produced a higher resistance to cytochalasin B than that by transfection of green fluorescent protein fusion h2-calponin in NIH 3T3 fibroblast cells (12). Based on the assumption that 3T3 cells had no endogenous h2-calponin, untransfected 3T3 control was not included in that study for comparison (12). However, we have found that NIH 3T3 cells express a significant level of h2-calponin (Fig. 9A). In the presence of endogenous h2-calponin, the cells overexpressing green fluorescent protein fusion h2-calponin retained most stress fibers after treatment with 2.5 μM (1.2 μg/ml) cytochalasin B for 15 min (see Fig. 5 in Ref. 12). This actually reflects a significant resistance, and therefore, the previous results may indicate that both h1 and h2 calponins could confer a resistance to cytochalasin B treatment (Fig. 8). The notion that h1 and h2 calponins may have conserved function is consistent with another previous study that transfective expression of h1-calponin had effects on the proliferation rate of NIH 3T3 cells (71) similar to the results of forced expression of h2-calponin in SM3 cells (13).

Tropomyosin is a major component in the actin cytoskeleton (43) and a protein that binds calponin (1, 20). The co-localization of h2-calponin and tropomyosin in the actin stress fibers (Fig. 7) and the positive correlation between the levels of force-expressed h2-calponin and endogenous tropomyosin in SM3 cells (Fig. 8C) suggest a functional relationship. Tropomyosin has been shown to play a role in actin fila-
Mechanical Tension Regulates h2-calponin

FIGURE 10. Mechanical tension-independent expression of viral promoter-directed h2-calponin transgene. A, time course of h2-calponin levels in h2-calponin sense cDNA-transfected SM3 cells after passage. The results are summarized from densitometry of multiple Western blots using RAH2 antibody on total protein extracts from two original stable transfected cell lines. In contrast to the post-cell roundup decrease of endogenous h2-calponin in keratinocytes and fibroblasts, the level of CMV promoter force-expressed h2-calponin in SM3 cells was not decreased but slightly increased at 12 and 24 h after cell passage (p < 0.01). B, h2-calponin sense cDNA-transfected SM3 cells were cultured on plastic dishes with or without continuous vibration that prevented cell attachment on the dish. Three-day-old floating cells were further cultured without vibration to form a monolayer. The cells were harvested at each of the states for Western blot analysis using RAH2 antibody to examine the levels of h2-calponin. The sample loading was normalized by their actin bands on SDS-gel and Western blot using RAH2 antibody on total protein extracts from two original stable transfected cell lines. In contrast to the post-cell roundup decrease of endogenous h2-calponin in the floating cells (*, p < 0.05), C, transfected SM3 cells cultured for 3 days on plastic dishes or soft polyacrylamide gels were examined by SDS-gel and Western blot using RAH2 antibody for the level of h2-calponin. Quantified by densitometry and normalized by the actin bands on parallel gel, the results showed a higher level of h2-calponin in the soft gel cultures than that of the plastic dish control (*, p < 0.001). The results for the cell anchorage and matrix stiffness experiments were each summarized from four individual experiments.

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