Published in final edited form as:

J Dermatol Sci. 2014 February ; 73(2): 142–151. doi:10.1016/j.jdermsci.2013.09.007.

Obligatory roles of filamin A in E-cadherin-mediated cell-cell adhesion in epidermal keratinocytes

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Abstract

Background—Extracellular Ca^{2+} (Ca^{2+}_o)-induced E-cadherin-mediated cell-cell adhesion plays a critical role in promoting differentiation in epidermal keratinocytes. Our previous studies show that the calcium-sensing receptor (CaR) regulates keratinocyte cell-cell adhesion and differentiation via Rho A-mediated signaling. CaR forms a protein complex with Rho A, guanine nucleotide exchange factor Trio, and a cytoskeletal actin-binding protein, filamin A, at the cell-cell junctions in response to elevated Ca^{2+}_o levels. Filamin A has the ability to interact directly with CaR, Trio, and Rho and mediate CaR-dependent signaling events.

Objective—This study was conducted to investigate the roles of filamin A and Trio in regulating Ca^{2+}_o -induced Rho activation and intercellular adhesion.

Methods—Expression of filamin A and Trio in keratinocytes was inhibited by siRNA. Its effects on Ca^{2+}_o -dependent junction formation and adhesion complex formation were evaluated by fluorescence immunostaining and immunoprecipitation. Endogenous Rho activity and expression of keratinocyte differentiation markers were also examined. The significance of the physical interactions of filamin A with Trio and Rho was assessed in dominant-negative inhibition studies.

Results—Inhibiting filamin A expression blocked the formation of CaR-Rho A-Trio-E-cadherin protein complex. Knockdown of filamin A or Trio inhibited Ca^{2+}_o -induced membrane localization and activation of Rho A, formation of the E-cadherin-catenin adhesion complex, and keratinocyte terminal differentiation. Expressing dominant-negative peptides disruptive to the endogenous filamin-Trio, filamin-Rho, and CaR-filamin interactions suppressed the formation of adherens junctions.

Conclusion—Through physical interactions with CaR, Trio and Rho, filamin A generates a scaffold for organizing a signaling complex that promotes E-cadherin-mediated cell-cell adhesion and keratinocyte differentiation.

Introduction

Extracellular Ca^{2+} (Ca^{2+}_o) promotes cell differentiation in epidermal keratinocytes by raising intracellular free Ca^{2+} levels and initiating intercellular adhesion [1, 2]. E-cadherin-

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The authors have no conflict of interest to declare

mediated cell-cell adhesion plays a key role in maintaining keratinocyte differentiation and epithelium tissue integrity [3, 4]. E-cadherin exerts its adhesive function by associating with cytoskeletal actin via catenins to form the adherens junction (AJ) [5, 6]. The E-cadherin-catenin adhesion complex recruits phosphatidylinositol 3-kinase (PI3K) and downstream effectors Akt and PLC γ 1 to the cell membrane, promoting cell differentiation and survival [2, 7].

E-cadherin-dependent cell-cell adhesion is regulated by the Rho family of small GTPases and the Src family of tyrosine kinases, especially Fyn [8, 9]. In keratinocytes, GTPases Rho and Rac are required for AJ formation [10]. While Rac may mediate actin recruitment, Rho is thought to take part in the clustering of cadherin at the cell-cell contacts [10, 11]. Inhibition of Rho A signaling impedes keratinocyte cell-cell adhesion [9] and terminal differentiation [12, 13]. Previous studies indicate that the Ca²⁺-sensing receptor (CaR) [14], a member of family C of the G-protein coupled receptor (GPCR) superfamily, regulates critical steps in E-cadherin-mediated cell-cell adhesion through the Rho/Fyn-mediated signaling pathway [13, 15]. Inhibiting CaR expression blocks the Ca²⁺_o-induced membrane translocation and activation of Rho A and Fyn, the formation of the E-cadherin-catenin complex, activation of PI3K and, consequently, keratinocyte differentiation [13, 15]. How CaR transduces Ca²⁺_o signals to activate the downstream Rho pathway is unclear. Evidence shows that the instigation of several CaR-mediated signaling events requires the involvement of an actin-binding protein, filamin A [16–18]. In keratinocytes, Ca²⁺_o promotes interaction between CaR and filamin A [13].

Filamin A is a ubiquitously expressed actin filament cross-linking protein with an actin-binding domain at the N-terminus, a dimerization domain at the C-terminus and a backbone of 24 immunoglobulin-like repeats. Filamin A is known to interact directly with a number of intracellular signaling proteins, ion channels, and transmembrane receptors including a subset of GPCRs [19, 20]. By coordinating the action of its binding partners, filamin is implicated in a number of cellular functions including cell motility, adhesion, receptor signaling, membrane ion channel activation, and protein stabilization and trafficking [21–23]. Filamin A binds to the carboxyl-terminal tail of CaR. This physical interaction between CaR and filamin is necessary for the localization of CaR to the cell membrane [23] and for CaR-mediated signaling to mitogen-activated protein kinase [18, 24, 25] and Rho [16, 17]. *In vitro* protein-binding assays also demonstrate direct interactions of filamin A with Rho-like GTPases and a guanine nucleotide exchange factor (GEF) for Rho-GTPases, Trio [26, 27]. Trio is a Dbl-family protein that contains two GEF domains (GEFD1 and GEFD2). TrioGEFD1 specifically activates Rac 1 and Rho G, while TrioGEFD2 targets Rho A [28, 29]. Each TrioGEFD contains a catalytic Dbl-homology (DH) domain followed by a pleckstrin-homology (PH) domain. TrioGEFD1 binds to filamin A through its PH domain, and such physical interaction is essential for GEFD1-mediated induction of actin cytoskeleton remodeling [27, 29]. These observations indicate that filamin functions as a scaffold for the spatial organization of Rho-GTPase-mediated signaling pathways.

In this study, we investigated the impacts of filamin A and Trio knockdowns on Ca²⁺_o-induced Rho activation, adhesion complex formation and keratinocyte differentiation. The importance of the physical interactions of filamin A with Trio and Rho on E-cadherin-dependent cell-cell adhesion was also assessed.

Materials and Methods

Cells and siRNA-mediated gene silencing

Human neonatal foreskin keratinocytes (NHKs) were cultured in serum-free growth medium (154CF, Cascade Biologics, Portland, OR) containing 0.03 mM CaCl₂ and used in the

experiments described. Pre-confluent keratinocytes were transfected with 20 nM siRNA (siGENOME SMART pool; Dharmacon Inc., Lafayette, CO) using PepMute siRNA transfection reagent (SignaGen Laboratories, Ijamsville, MD). Three days later cells were exposed to 2 mM CaCl_2 for 10 minutes to induce the formation of cell-cell contacts, or to 1.2 mM CaCl_2 for 72 hours to induce differentiation. The committee for Human Research at the San Francisco Veteran Affairs Medical Center and the University of California San Francisco approved the use of human keratinocytes.

Antibody

The following antibodies (Abs) were used in this study: monoclonal antibodies (mAbs) for $\alpha 2$ integrin (BD Biosciences, San Jose, CA), mAbs for involucrin and β -actin, c-Myc-, V5- and HA-tags (Sigma-Aldrich, St. Louis, MO), polyclonal and monoclonal Abs against E-cadherin, and β -, γ -, and p120-catenin, polyclonal Abs against Rho A, Rac1 and Trio, mAb against transglutaminase 1 (Santa Cruz Biotechnology, Santa Cruz, CA), mAbs for Rho A and filamin A (Millipore Corp., Temecula, CA), polyclonal Abs for human keratin 1, keratin 14, filaggrin and loricrin (Covance Inc., Berkeley, CA), and rabbit anti-human CaR [30].

Generation of recombinant adenoviruses and infection of keratinocytes

The cDNAs encoding the PH domains in Trio GEFD1 and GEFD2 and the filamin A repeat 23–24 were amplified from human keratinocytes by PCR using specific primers (see supplementary Figure S2). The cDNAs encoding the CaR C-terminal tail and the filamin A repeats 15–16 were produced as previously described [13]. Replication-defective adenoviruses expressing these cDNAs were constructed using an Adeno-X Expression System II kit (BD Biosciences) and produced in HEK293 cells. Sub-confluent NHKs were infected with adenoviruses at a titer of 100 pfu/cell in growth medium containing 0.03 mM CaCl_2 , then cultured for 5 days before exposure to 2 mM CaCl_2 for 10 minutes to induce the formation of cell-cell contacts.

Rho and Rac activity

NHKs were exposed to 2 mM Ca^{2+} for 10 minutes and then lysed in MLB buffer (1% NP-40, 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl_2 , 1 mM EDTA, 1 mM Na_3VO_4 , 20 mM NaF) supplemented with protease inhibitors and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN). Cell lysates containing 800 μg total protein were incubated with 20 μg of agarose-conjugated GST-tagged fusion protein containing Rhotekin Rho binding domain or PAK-1 p21-binding domain (Millipore Corp.), which respectively binds specifically to GTP-Rho or GTP-Rac, in cold MLB buffer at 4°C overnight. Precipitates were washed, eluted, then evaluated by immunoblotting analysis for Rho A or Rac1, respectively.

Dispase dissociation assay

Triplicate wells of confluent keratinocyte cultures in medium containing either 0.03 or 2 mM CaCl_2 were washed in PBS and incubated in 2.5 U/ml dispase solution (BD Biosciences) at 37°C for 15 min. Cells from each sample, including single cells released into media and detached cell sheets, were collected by scraping, and then washed with PBS. After centrifugation, keratinocytes were resuspended in PBS by pipetting 15 times with 1 ml pipet tips. The number of single cells in suspension was counted using a hemocytometer. The whole samples were then centrifuged and incubated with 0.05% trypsin at 37°C for 10 min to release all cells into suspension. The number of single cells released by dispase was normalized to total cells. Student's t-test and one-way ANOVA were performed to analyze the data. The statistical difference is determined significant if the P value is lower than 0.05.

Cell lysate preparation, immunoblotting and immunoprecipitation

The conditions for preparing total keratinocyte lysates and membrane proteins, immunoblotting and immunoprecipitation were as described previously [15]. 50 µg protein samples were used in immunoblotting analyses. Cell lysate containing 750 µg total protein or 200 µg membrane protein were immunoprecipitated by 3 µg of designated antibodies, followed by Sepharoseconjugated protein G (Thermo Fisher Scientific, Rockford, IL).

Fluorescence immunostaining

Keratinocytes were cultured on coverslips, fixed with 4% paraformaldehyde, and permeabilized with 0.5% NP-40 in PBS. Conditions for staining were as described previously [15]. After incubation with 10 µg/ml of primary antibodies and, subsequently, with 10 µg/ml of the appropriate fluorescein- or Texas redconjugated secondary antibody (Invitrogen Corp.), coverslips were mounted and examined with a Leica TCS NT/SP confocal microscope (Leica Microsystems, Heidelberg, Germany).

Results

Inhibition of filamin A and Trio expression suppressed E-cadherin-mediated cell-cell adhesion and keratinocyte differentiation

Fluorescence immunostaining showed that elevating $[Ca^{2+}]_o$ from 0.03 to 2 mM promoted the translocation of Rho A (Fig 1A), CaR (Fig 1B), filamin A (Fig 1C), and Trio (Fig 1E) with E-cadherin from the cytosol to cell-cell junctions in human epidermal keratinocytes. Ca^{2+}_o also stimulated the association of CaR (Fig 1D) and Trio (Fig 1F) with filamin A at the cell membrane. These observations were consistent with the notion that filamin is involved in organizing a signaling protein complex with CaR, Rho-GEF Trio, and Rho A at the cell membrane, eliciting the E-cadherin-mediated cell-cell adhesion in response to Ca^{2+}_o . To delineate the function of filamin and Trio, we inhibited their expressions in keratinocytes with siRNAs specifically targeting filamin A (siFLNA) and Trio (siTrio). Immunoblotting analyses of total cell lysates from siRNA-transfected keratinocytes showed that endogenous filamin A and Trio levels were effectively reduced by siFLNA and siTrio, respectively, as compared with the cells transfected with control siRNA (siControl) (Fig 2A). Meanwhile neither Ca^{2+} (2 mM) nor gene knockdown altered the total expression levels of Rho A, E-cadherin, β -catenin, or γ -catenin.

To determine whether inhibition of filamin A affects the formation of the signaling complex required for E-cadherin-mediated adhesion, we immunoprecipitated plasma membrane lysates using a Rho A antibody followed by protein G-conjugated sepharose beads. Analyses of immunoprecipitates showed that Ca^{2+}_o increased the protein level of Rho A in the cell membrane as well as the amount of associated CaR, filamin A, Trio, and E-cadherin in control keratinocytes (Fig 2B). Inhibiting filamin A expression by siFLNA suppressed the Ca^{2+}_o -stimulated membrane expression of Rho A and, therefore, the formation of the CaR-Rho A-Trio-E-cadherin complex (Fig 2B). We then examined the impact of filamin and Trio knockdowns on endogenous Rho activity in response to Ca^{2+}_o . Levels of the activated form of Rho (Rho-GTP) in total cell lysates were determined by pull-down assays using a fusion protein containing the rhotekin Rho-binding domain. As shown in Fig 2C, elevating $[Ca^{2+}]_o$ from 0.03 to 2 mM increased the Rho-GTP levels in control keratinocytes, whereas inhibiting the expression of filamin or Trio by siFLNA or siTrio, respectively, blocked the ability of Ca^{2+}_o to increase endogenous Rho activity. In contrast, neither gene knockdown nor Ca^{2+} treatment affected the total Rho A level (Fig 2C). Fluorescence immunostaining demonstrated that Ca^{2+}_o induced colocalization of β -catenin (Fig 3A) and Rho A (Fig 3B) with E-cadherin at the cell-cell junctions in control keratinocytes, while inhibiting the expression of filamin A or Trio suppressed Ca^{2+}_o -induced intercellular adhesion. Similar to

keratinocytes that lost the ability to form AJs due to a lack of either CaR or Rho A [13, 30], siFLNA- and siTrio-transfected keratinocytes appeared larger in size, with glossier surfaces and plumper shapes than control cells (Fig 3 and unpublished observations).

We next examined the levels of the E-cadherin-catenin adhesion complex in these cells by co-immunoprecipitation. Plasma membrane lysates from siRNA- treated keratinocytes were precipitated with an antibody against E-cadherin (Fig 4A), followed by protein G-conjugated sepharose beads. The immunoprecipitates were then analyzed for the presence of the components of the adhesion complex by immunoblotting. In low Ca^{2+} (0.03 mM) conditions, small amounts of β -, γ - and p120-catenins associated with E-cadherin in the cell membranes of control keratinocytes. Ca^{2+} (2 mM) treatment stimulated the association of Rho A with E-cadherin and increased the occurrence of the E-cadherin-catenin complex (Fig 4A). Knocking down filamin A or Trio markedly reduced the levels of E-cadherin and catenins in the cell membrane (Fig 4A), signifying the importance of filamin and Trio in forming the adhesion complexes. Similar results were observed when plasma membrane lysates were precipitated with a Rho A antibody (Fig 4B). Ca^{2+} increased the amount of E-cadherin-catenin-Rho complex in the cell membrane of control keratinocytes, but this was noticeably diminished in the siFLNA- and siTrio-transfected cells, likely due to decreased membrane Rho A (Fig 4B). Immunoblotting analyses of the plasma membrane lysates (Supplementary Fig S1) verified that the ability of Ca^{2+} to increase the localization of Trio, Rho A, and E-cadherin in the cell membrane in control keratinocytes was blocked when filamin A expression was inhibited by siFLNA.

To assess whether the biochemical changes in cell-cell junctions resulting from filamin and Trio inhibition cause any functional defects, we evaluated the cohesiveness of cell adhesion with a dispase dissociation assay. With a lack of cell-cell adhesion, confluent keratinocytes cultured in 0.03 mM Ca^{2+} were susceptible to dispase treatment, causing detachment of most keratinocytes (> 95%) as single cell suspensions (Fig 4C). Prolonged exposure of keratinocytes to 2 mM Ca^{2+} promoted the formation of cell-cell contacts and increased their resistance to dispase digestion (Fig 4C). As shown in Fig 4C, inhibiting either filamin A or Trio resulted in a significant increase in the number of dispase-released single cells as compared to controls. Treating keratinocytes with 2 mM Ca^{2+} for 60 min resulted in 54% of control keratinocytes released as single cells, versus 78% of siFLNA- and 73% of siTrio-transfected cells. Although keratinocytes had formed even stronger intercellular junctions after 120 min of calcium treatment, inhibiting filamin A and Trio expression still caused a considerable reduction in the strength of cell adhesion (Fig 4C; siFLNA- and siTrio vs. siControltransfected cells, 57% and 47% vs. 26% released by dispase, $P < 0.01$).

To investigate whether knockdown of filamin or Trio affect cell differentiation, we compared the expression of terminal differentiation genes in siRNA-treated keratinocytes after a 72-hr exposure to 1.2 mM Ca^{2+} . Immunoblotting analyses of total lysates (Fig 4D) demonstrated that Ca^{2+} -augmented expression of the terminal differentiation marker proteins keratin 1, involucrin, transglutaminase (TG) 1, loricrin and filaggrin were substantially decreased in siFLNA- and siTrio-treated keratinocytes as compared to control cells. Neither Ca^{2+} nor gene knockdown altered the protein level of the basal keratinocyte marker keratin 14. These results clearly indicate that filamin A and Trio are essential for keratinocyte cell-cell adhesion and differentiation.

Ca^{2+} -activation of Rho A and E-cadherin-mediated cell-cell adhesion required filamin-Trio and filamin-Rho interactions

Several studies have demonstrated that filamin A interacts directly with CaR, Trio and Rho family GTPases. The mid-portion of the CaR carboxyl-terminus tail (amino acids 906–980) interacts with the region of filamin A that contains repeats 14–17 and hinge 1 (amino acids

1530–1875) [18, 23, 24]. *In vitro* binding assays have shown that TrioGEFD1 binds to filamin repeats 23–24 (amino acids 2354–2647) via its PH domain (amino acids 1407–1628) [27]. Filamin A also interacts with several Rho-like small GTPases, including Rho A and Rac1, possibly through its carboxyl-terminus repeats [26].

We hypothesize that filamin A provides a scaffold to aid in the assembly of a signaling protein complex that activates E-cadherin signaling and promotes cell-cell adhesion through its interactions with Trio, Rho A, and CaR. Here we investigated whether disrupting the filamin-Trio and/or the filamin-Rho interactions affects the Ca^{2+}_o -induced Rho activation and cell-cell adhesion. Three peptides were used to compete with endogenous filamin A, Trio, and Rho for their respective native binding partners (Supplementary Fig S2). To disrupt the endogenous filamin-Trio interaction, keratinocytes were separately infected with adenoviruses expressing a HA-tagged Trio peptide containing GEFD1 PH domain (TrioPH1, amino acid 1423–1533), a Myc-tagged Trio peptide containing GEFD2 PH domain (TrioPH2, amino acid 2101–2214), or a V5-tagged filamin A peptide containing repeats 23–24 (FLNaT, amino acid 2177–2647). Since the Trio-binding domain in filamin overlaps with the region that interacts with Rho-like GTPases, over-expressing these dominant-negative peptides may also interfere with the filamin-Rho interaction. Adenovirus without a cDNA insert was used as a control. Immunoprecipitation of either TrioPH1-expressing keratinocyte lysates using an anti-HA antibody or TrioPH2-expressing keratinocyte lysates using an anti-Myc antibody brought down filamin A, whereas an anti-V5 antibody pulled down Trio in FLNaT-expressing cell lysates (Supplementary Fig S3). These results confirmed direct interactions of these peptides with their respective native binding partners.

To test whether Ca^{2+}_o -induced Rho activation requires the physical interactions of filamin with Trio and Rho, TrioPH1-, TrioPH2- and FLNaT-expressing keratinocytes were exposed to 2 mM Ca^{2+}_o for 10 min, then Rho activity in total cell extracts was determined based on the level of GTP-bound Rho A (Rho-GTP). As shown in Fig 5A, interrupting the filamin-Trio interaction with TrioPH1, TrioPH2 and FLNaT inhibited the ability of Ca^{2+}_o to increase endogenous Rho A activity. Neither treating with Ca^{2+}_o nor expressing Trio or filamin peptides changed the total expression level of Rho A. Since the PH domain in TrioGEFD1 is responsible for filamin binding and directing Trio to its downstream targets, including both Rho A and Rac1, disrupting the filamin-Trio interaction with these dominant-negative peptides may also affect the endogenous Rac activity. As shown in Fig 5B, Ca^{2+}_o increased the level of active Rac1 (Rac-GTP) in keratinocytes. Expressing either TrioPH1 or FLNaT blocked the induction of Rac1 by Ca^{2+}_o , validating the importance of the TrioGEFD1 PH domain in the activation of Rho-GTPases. On the contrary, expressing TrioPH2 had no effect on the Ca^{2+}_o activation of Rac1 (Fig 5B), indicating that the PH domain in TrioGEFD2 is not involved in regulating Rac signaling.

To assess whether TrioPH1, TrioPH2, and FLNaT peptides disturb the interactions of endogenous filamin with Trio and Rho, we immunoprecipitated plasma membrane lysates using an antibody against Trio (Fig 6A). Immunoblotting analyses of immunoprecipitates for filamin A showed that the ability of Ca^{2+}_o to promote the association of Trio and filamin A in the cell membrane in control keratinocytes was inhibited, with varying efficacy, by TrioPH1, TrioPH2 and FLNaT (Fig 6A), indicating of a perturbed endogenous filamin-Trio interaction. Expressing TrioPH1, TrioPH2, and FLNaT also diminished the Ca^{2+}_o -stimulated membrane recruitment of Trio and Rho A, and reduced the levels of E-cadherin-catenin complex in the cell membrane (Fig 6A). Comparable results were obtained when the plasma membrane lysates were immunoprecipitated with a Rho A antibody (Fig 6B). Immunoblotting analyses of immunoprecipitates for filamin A showed that Ca^{2+}_o -promoted association of Rho A and filamin at the cell membrane was reduced by TrioPH1, FLNaT,

and, to a lesser extent, TrioPH2 (Fig 6B), likely due to decreased Rho A levels in membrane. Fluorescence immunostaining showed that TrioPH1, TrioPH2 or FLNaT inhibited the ability of Ca^{2+}_o to induce the formation of adherens junctions (Figure 6C) and the colocalization of Rho A with E-cadherin (Figure 6D) at the cell membrane. We then evaluated the strength of intercellular adhesion in keratinocytes expressing these dominant-negative peptides using disperse dissociation assays. Disrupting the interactions of filamin with Trio and Rho significantly increased the susceptibility of cell-cell junctions to disperse and increased the number of single cells (Fig 6E; TrioPH1-, TrioPH2- and FLNaT-expressing vs. control keratinocytes, 90%, 76% and 77% vs. 60% after a 60-min calcium treatment, 65%, 43% and 51% vs. 25% after a 120-min calcium treatment, * $P < 0.01$, # $P < 0.05$).

Similarly, disrupting the endogenous CaR-filamin interaction by infecting keratinocytes with adenoviruses that express either a CaR C-terminus peptide (CaRL, amino acid 907–980) or a filamin A peptide containing repeats 15–16 (FLNaL, amino acid 1566–1875) inhibited the ability of Ca^{2+}_o to increase endogenous Rho activity and attenuated keratinocyte cell-cell adhesion (Supplementary Fig S4). These results demonstrated that Ca^{2+}_o -activation of Rho A and E-cadherin-mediated cell-cell adhesion required the physical interactions of filamin with the RhoGEF Trio, Rho-GTPase and CaR.

Discussion

Cell-cell adhesion is regulated by complex processes that involve cytoskeletal actin, adhesion proteins and an array of intracellular signaling molecules. Trio is a member of the DH-GEF family that regulates cytoskeletal reorganization and cell adhesion via activation of Rho-GTPases. Trio and Rac1 form a protein complex with M-cadherin at the onset of myoblast fusion [31]. Silencing Trio expression *in vitro* reduces Rac1 activity and prevents myotube formation [31]. Trio-deficient mouse embryos display defective skeletal muscle formation and aberrant organization of brain tissues [32], resulting from a loss of Trio-mediated activation of Rac1 that promotes cytoskeletal actin rearrangement and cell migration [31, 33].

Although Trio likely regulates E-cadherin-mediated cell-cell adhesion in keratinocytes through Rho A activation, Rac signaling may take part in these processes as well, since raising Ca^{2+}_o levels increased endogenous Rac1 activities (Fig 5B). The PH domain in TrioGEFD1 (TrioPH1) is essential for Rac1-mediated Jun kinase activation and cytoskeletal remodeling in fibroblasts and for Trio-induced neurite outgrowth in the PC12 cells [29]. Whereas TrioPH1 localizes Trio near its downstream targets through interaction with filamin [27] and facilitates the catalytic ability of its associated DH domain by directly engaging Rho-GTPases [34], the function of the PH domain in TrioGEFD2 (TrioPH2) is unclear. In keratinocytes, peptides expressing either TrioPH1 or repeats 23–24 of filamin A blocked not only Ca^{2+}_o -activation of Rho A (Fig 5A), but also the induction of Rac1 (Fig 5B), confirming the importance of the interaction between TrioPH1 and filamin in the activation of Rho-GTPases. In contrast, expressing TrioPH2 peptide inhibited Rho A activation, while it had no effect on Rac1 (Fig 5B), even though both TrioPH1 and TrioPH2 peptides independently interacted with endogenous filamin (Supplementary Fig S3). These results indicated a selective role for the TrioPH2 domain in regulation of Rho A signaling. Although Trio is a key mediator in Rho signaling and E-cadherin-mediated cell-cell adhesion in keratinocytes, our observations do not preclude the involvement of other GEFs. For example, RhoGEF Lbc physically interacts with CaR, Rho A, Gαq and filamin, and participates in the CaR-dependent Rho-mediated transcriptional activation of serum response element [17].

A role for filamin A in cell-cell adhesion was first noted in the filamin A (Flna)-null mice [35] that had reduced or absent VE-cadherin surface expression in vascular vessel endothelial cells and in neuroepithelial cells at the ventricular surface. These mice manifest severe cardiac structural defects and aberrant vasculature due to abnormal AJ formation [35]. Surprisingly, the actin cytoskeletal structure, cell motility and migration were normal in a variety of cell types in Flna-null mice, despite the importance of filamin A in maintaining these cellular functions [35]. Although the molecular mechanism for filamin's regulation of vascular endothelial cell-cell adhesion requires elucidation, filamin A may connect membrane adhesion molecules to intracellular cytoskeletal complexes or function as a molecular switch to regulate actin dynamics at AJs. Loss-of-function mutations at the human FLNA locus are identified as the genetic cause of EhlersDanlos variant of periventricular heterotopia (EDS-PH) [36]. In addition to brain malformation as a result of abnormal neuronal migration along the ventricular lining, the EDS-PH patients exhibit a wide spectrum of vascular and connective anomalies including vascular fragility, joint hypermobility, skin hyperelasticity, skin fragility and scarring. Filamin A is known to bind integrins and membrane glycoproteins that mediate the cell-extracellular matrix (ECM) adhesion [19, 20]. Decrease in the strength of attachment of the cells to ECM or impaired regulation of the cytoskeleton through altered filamin-integrin interaction may be factors in the pathogenesis of EDS-PH.

Our studies have demonstrated the contribution of filamin A to E-cadherin-mediated cell-cell adhesion. Raising Ca^{2+}_o levels augmented the membrane expression of CaR, filamin A, Trio, Rho A, and E-cadherin (Fig 1 and Supplementary Fig S1), and promoted complex formation of these proteins (Fig 2B) and the formation of AJs. Inhibiting filamin A expression blocked these Ca^{2+}_o -dependent events. These results signified the importance of filamin A in recruiting cadherin and effectors in Rho-GTPase signaling to the intercellular junctions, facilitating the actin cytoskeleton remodeling required for establishing stable cell-cell adhesion. Nevertheless, inhibiting Trio expression or disrupting filamin-Trio interaction blocked Ca^{2+}_o -induced membrane localization (Fig 4B, 6B, 6D, and Supplementary Fig S1) and activation (Fig 2C and Fig 5A) of Rho A, indicating the necessity of Trio for these processes. It is conceivable that the interaction of Trio with filamin may facilitate the membrane recruitment of Rho-GTPase and their activation, as the Trio-binding and Rho-binding domains in filamin are in close proximity.

Ca^{2+}_o -activation of Rho requires the physical interaction of the C-terminal region of CaR with filamin A (Supplementary Fig S4 and reference [13]), as well as the interactions of the Trio PH domains and Rho-GTPase with the C-terminus repeats of filamin A (Fig 5). Disrupting either the CaR-filamin or filamin-Trio interaction in keratinocytes by expressing dominant-negative CaR, Trio, and filamin peptides disconnected the CaR-dependent transduction of Ca^{2+}_o signals to E-cadherin-mediated cell-cell adhesion. Our results indicated that filamin A creates a scaffold for the spatial organization of a signaling complex through physical interactions with CaR, Trio and Rho, activating E-cadherin pathway and promoting cell differentiation (Fig 7).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Sally Pennypacker and the Cell Culture and Tissue Preparation Core for preparing human epidermal keratinocytes. We appreciate the editorial assistance from Rustin Zomorodi for preparing this manuscript.

Funding statement

This work was supported by grant RO1-AR056256 from the National Institutes of Health, USA.

Abbreviation

Ca²⁺_o	extracellular Ca ²⁺
CaR	calcium-sensing receptor
AJ	adherens junction
PI3K	phosphatidylinositol 3-kinase
siRNA	small interfering RNA
GEF	guanine nucleotide exchange factor

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Fig 1. Elevated $[Ca^{2+}]_o$ induced colocalization of Rho A, CaR, filamin A and E-cadherin at the cell-cell junctions. Keratinocytes were exposed to 2 mM Ca^{2+} for 10 minutes to induce cell-cell contacts. Fluorescence immunostaining was performed using antibodies against Rho A (A), CaR (B, D), Trio (E, F), filamin A (C, D, F) and E-cadherin (A, B, C, E), followed by FITC-conjugated (green) and Texas redconjugated (red) secondary antibodies. Overlapped staining sites are visualized as yellow. Bar = 20 μ m (A, B), 10 μ m (C, D, E), or 5 μ m (F).

Fig 2. Inhibition of filamin A expression by siRNA blocked the Ca^{2+}_o -induced formation of a signaling protein complex at the cell membrane and activation of Rho. Keratinocytes were transfected with filamin A-specific (siFLNA), Trio-specific (siTrio) or control siRNA (siControl) and then exposed to 2 mM Ca^{2+} for 10 minutes. (A) Protein levels of filamin A, Trio, Rho A, E-cadherin, and β - and γ -catenin were examined by immunoblotting analyses of total cell lysates. β -actin was used as a loading control. (B) Plasma membrane lysates of siFLNA or siControl-transfected keratinocytes were immunoprecipitated (IP) with an antibody against Rho A. The immunoprecipitates were analyzed by immunoblotting for CaR, Trio, Rho A, filamin A, and E-cadherin. $\alpha 2$ -integrin was used as a control for equal input of plasma membrane protein before precipitation. (C) Activated Rho (Rho-GTP) in cell lysates was pulled down by agarose beads conjugated to the Rho-binding domain of rhotekin and then analyzed by immunoblotting for Rho A. Corresponding total cell extracts were analyzed for total Rho A protein levels. β -actin was used as a control for equal protein input before pull-down.

Fig 3. Inhibition of filamin A or Trio expression suppressed Ca^{2+}_o -induced colocalization of β -catenin, Rho A and E-cadherin at the cell-cell junctions. SiFLNA-, siTrio- or siControl-transfected keratinocytes were exposed to 2 mM Ca^{2+} for 10 minutes to induce cell-cell adhesion. Fluorescence immunostaining was performed using antibodies against β -catenin (A), Rho A (B), and E-cadherin (A, B) followed by FITC- and Texas red-conjugated secondary antibodies. Overlapped staining sites are visualized as yellow. Bar= 20 μm .

Fig 4. Inhibition of filamin A or Trio expression suppressed keratinocyte cell-cell adhesion. SiFLNA-, siTrio- or siControl-transfected keratinocytes were exposed to 2 mM Ca²⁺ for 10 minutes. Plasma membrane lysates were immunoprecipitated (IP) with E-cadherin (A) or Rho A (B) antibodies. Immunoprecipitates were analyzed by immunoblotting for E-cadherin, β-, γ-, p120-catenin, and Rho A. α2-integrin was a control for equal protein input before precipitation. (C) Confluent keratinocytes cultured in 0.03 or 2 mM Ca²⁺ were treated with dispase as described in Materials and Methods. Disperse-released single cells were normalized to total cells. The data are presented as mean ± SD. *P<0.01, siFLNA- and siTrio- vs. siControl-treated keratinocytes. (D) SiRNA-treated keratinocytes were cultured in medium containing 0.03 or 1.2 mM Ca²⁺ for 72 hours. Cell lysates were analyzed by immunoblotting for differentiation markers keratin 1, involucrin, transglutaminase 1 (TG1), loricrin, filaggrin, and basal cell marker keratin 14.

Fig 5.

Expressing dominate-negative peptides that disrupt filamin-Trio and filamin-Rho interactions inhibited Ca²⁺_o-induced activation of Rho A and Rac1. Keratinocytes were infected by adenoviruses expressing the Trio GEFD1 PH (TrioPH1), Trio GEFD2 PH (TrioPH2) and filamin A domains 23–24 (FLNaT) to disrupt filamin-Trio and filamin-Rho interaction. Adenoviruses without cDNA inserts were used as controls. Virus-infected keratinocytes were exposed to 2 mM Ca²⁺ for 10 minutes. (A) Levels of activated Rho A (Rho-GTP) in total cell lysates were determined as described in Fig 2. (B) Activated Rac (Rac-GTP) in cell lysates was pulled down by agarose beads conjugated to the Rac-binding domain of PAK-1, and then analyzed by immunoblotting for Rac1. Corresponding total cell extracts were analyzed for total Rac1 protein levels. β-actin was used as a control for equal protein input before pull-down.

Fig 6.

Disrupting filamin-Trio and filamin-Rho interactions decreased the Ca²⁺_o-induced formation of a protein complex containing filamin, Trio, Rho A and E-cadherin at the cell membrane and suppressed keratinocyte cell-cell adhesion. Keratinocytes were infected by adenoviruses expressing the Trio GEFD1 PH (TrioPH1), Trio GEFD2 PH (TrioPH2) and filamin A domains 23–24 (FLNaT) to disrupt filamin-Trio and filamin-Rho interaction, and then were exposed to 2 mM Ca²⁺ for 10 minutes. Plasma membrane lysates were immunoprecipitated (IP) with antibodies against Trio (A) or Rho A (B). The immunoprecipitates were analyzed by immunoblotting for Rho A, filamin A, Trio, E-cadherin and β- and γ-catenin. Fluorescent immunostaining was performed using antibodies against β-catenin (C), Rho A (D) and E-

cadherin (C, D), followed by the FITC- and Texas red-conjugated secondary antibody. Overlapped staining sites are visualized as yellow. Bar = 20 μ m. (E) Strength of intercellular adhesion was evaluated by dispase digestion as described in Fig 4. The data are presented as mean $\pm$ SD. *P<0.01, #P<0.05, TrioPH1-, TrioPH2- and FLNaT-expressing vs. control keratinocytes.

Fig 7.

Filamin A serves as a scaffold for the assembly of a signaling complex required for E-cadherin mediated cell-cell adhesion. In response to Ca stimulation, CaR, Trio, and Rho A form a signaling complex at the cell membrane to activate downstream effectors to promote the formation of the E-cadherin-catenin adhesion complex. Whereas Trio may serve as a guanine nucleotide exchange factor for Rho A, filamin provides a scaffold for the spatial organization of this signaling complex through direct protein-protein interactions with CaR, Trio and Rho-GTPase. Stabilized adhesion complexes recruit other signaling factors such as PI3K to promote keratinocyte differentiation.