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β-Adducin siRNA disruption of the spectrin-based cytoskeleton in differentiating keratinocytes prevented by calcium acting through calmodulin/epidermal growth factor receptor/cadherin pathway

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ABSTRACT

Here, we report that siRNA transfection of β-adducin significantly disrupted the spectrin-based cytoskeleton and cytoskeletal arrangements of both β-adducin and PKCδ by substantially inhibiting the expression of β-adducin, spectrin and PKCδ proteins in differentiating keratinocytes. However, extracellular Ca^{2+} treatment blocked the inhibitory effects of the β-adducin siRNA. Ca^{2+} also prevented the significant down-regulation of two differentiation markers involucrin and K1/10 and the distinct up-regulation of proliferation marker K14 in β-adducin siRNA transfected keratinocytes. In addition, β-adducin knockdown resulted in a substantial reduction of epidermal growth factor receptor (EGFR), cadherin and β-catenin and enhanced phosphorylation of EGFR on tyrosine 1173 and Ca^{2+} prevented these changes. Furthermore, Ca^{2+} blocked the inhibitory effects of β-adducin siRNA on the expression of calmodulin, phosphorylated-calmodulin (P-CaM$^{(Tyr138)}$) and myristoylated alanine-rich C-kinase substrate (MARCKS) in keratinocytes. Co-immunoprecipitation studies further revealed that calmodulin, not MARCKS, strongly interacted with EGFR, cadherin and β-catenin. Our data suggest that Ca^{2+} plays an important role in regulating the expression and function of β-adducin to sustain normal organization of the spectrin-based cytoskeleton and the differentiation properties in keratinocytes through the calmodulin/EGFR/cadherin signaling pathway.

1. Introduction

Keratinocyte constitutes 90% of the cells in the outermost layer of the skin in forming the protective barrier of mammalian skin [1]. Primary keratinocytes in in vitro cultures resemble in vivo epidermal development when they enter a differentiation program [2]. We and others have previously observed that both human and mouse primary keratinocytes grown in vitro proceed to cell differentiation with downregulation of proliferation markers including K14 and K5 and upregulation of differentiation markers such as involucrin and K10 [3–5]. Furthermore, the transcription factor Sp1 is also upregulated while transcription factor-1 (UTF-1) is downregulated during cell differentiation in primary mouse and human keratinocyte cultures [6–8]. Thus, the proliferation and differentiation capacity of cultured epidermal keratinocytes makes them ideal candidates for investigating the regulatory role of the genes involved in the development of the epidermis and the use of drug therapy in skin diseases [6,9].

Adducin is a ubiquitously expressed membrane-skeletal protein, which is localized to the plasma membrane of a variety of tissues [5]. This skeletal protein that functions as an F-actin capping protein is always observed in association with spectrin and actin in tissues and cultured cells [5,10]. Spectrins are ubiquitous scaffolding components of the membrane skeleton that organize and stabilize microdomains on both the plasma membrane and the intracellular organelles [11]. The spectrins are implicated in various cellular functions by way of their numerous interactions with diverse protein families [11]. In the presence of spectrin, adducin promotes the binding of this protein to actin to assemble a spectrin–actin lattice. A major mode of association of adducin and spectrin is in a ternary complex at the plus-ends of actin filaments [10,12]. Therefore, adducin is required to stabilize preformed lateral membrane of human bronchial epithelial cells through interaction with β2-spectrin [5]. Furthermore, as an in vivo substrate for protein kinase C (PKC) and Rho-associated kinase, adducin is regulated by these enzymes together with calmodulin to promote assembly of spectrin–actin complexes [10,13,14]. We have previously reported that β-adducin, together with spectrin and PKCδ, increases dramatically in amount and is assembled into the cytoskeletal organization during cell differentiation in both mouse and human primary keratinocytes,

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suggesting further that β-adducin plays an important role in keratinocyte differentiation [15,16].

As one of a small number of second messengers, Ca^{2+} has been used by mammalian cells to mediate the complex biological processes associated with cell proliferation and differentiation. In the skin, total and free Ca^{2+} are low in the basal keratinocyte and first suprabasal keratinocyte layers, but extraordinarily high in the granular keratinocyte layer where the keratinocytes proceed to terminal cell differentiation in mouse and human epidermis [12,17]. The existence of the Ca^{2+} gradient reveals its importance as a physiological regulator of epidermal development and differentiation [12]. Furthermore, as an enhancer of cell differentiation, Ca^{2+} promotes mouse keratinocyte differentiation in vitro, which regulates the expression of differentiation markers such as involucrin and K1/K10 and viral capsid proteins [18]. Ca^{2+} also plays roles in directional sensing, cytoskeleton redistribution, traction force generation, and relocation of focal adhesions in migrating cells [15,19].

It has been reported previously that siRNA depletion of α-adducin from confluent human bronchial epithelial cells results in increased detergent solubility of spectrin after normal membrane biogenesis during mitosis [5]. siRNA-mediated down-regulation of α- or γ-adducin expression significantly attenuates the assembly of calcium-dependent epithelial adherens junctions (AJ) and tight junctions (TJ) and accelerates junctional disassembly triggered by PKC activation [5]. We observed that siRNA transfection of β-adducin in differentiating keratinocytes results in significant reduction of not only β-adducin protein, but also spectrin and PKCδ proteins, leading to the disruption of the spectrin-based cytoskeleton and the abnormal cytoskeletal arrangements of both adducin and PKCδ in keratinocytes [16]. Previous studies have shown that the presence of adducin at cell–cell contact sites of cultured epithelial cells requires extracellular Ca^{2+} [20] and Ca^{2+}-dependent pathways regulate the role of adducin in cell motility [21]. However, it has not been reported whether Ca^{2+} plays any regulatory role in the expression and function of adducin in keratinocytes. Here we report that β-adducin siRNA transfection resulted in a significant reduction of β-adducin protein in keratinocytes, associated with a dramatic change in cell morphology. β-Adducin knockdown led to substantial disruption of the spectrin-based cytoskeleton accompanied by a significant decrease of both spectrin and PKCδ. We have also shown that β-adducin knockdown significantly downregulated the expression of differentiation marker proteins such as involucrin and K1/10, but a distinctly upregulated expression of the proliferation marker K14 and tubulin in keratinocytes suggesting that β-adducin knockdown resulted in abrogation of cell differentiation. We demonstrate that Ca^{2+} prevented the changes of expression of the targeted proteins and disruption of spectrin-based cytoskeleton caused by β-adducin siRNA transfection in keratinocytes through the Ca^{2+}-calmodulin/EGFR/cadherin signaling pathway.

2. Materials and methods

2.1. Antibodies

Primary antibodies including mouse monoclonal antibodies (Abs) to αI-spectrin and calmodulin and rabbit polyclonal Abs to PKCδ, β-adducin and p-β-adducin$^{(Ser662)}$, EGFR, p-EGFR$^{(Tyr1173)}$, p-calmodulin$^{(Tyr138)}$ and p-MARCKS$^{(Ser159)}$ and goat polyclonal Abs to βI spectrin, p-PKCδ$^{(Thr505)}$ and MARCKS were purchased from Santa Cruz (USA). Primary antibodies (Abs) from Sigma-Aldrich (Australia) were rabbit polyclonal Abs to human spectrin and to actin; and mouse monoclonal Ab to β-tubulin. Rabbit monoclonal Abs to E-cadherin, β-catenin, Src, p-Src$^{(Y527)}$, PI3K-p85 and p-PI3K-p85 were purchased from Cell Signalling (USA). Rabbit polyclonal Ab to involucrin was purchased from Covance (USA). Mouse monoclonal Ab to IFN-γ was purchased from MABTCH (Australia).

2.2. Control and β-adducin siRNAs

Both control siRNA (SC-37007; St Cruz, USA) and β-adducin siRNA (SC-37061, St Cruz, USA) were purchased from St Cruz, USA. The β-adducin siRNA consists of pools of three to five target-specific 19–25 nt siRNAs designed to knockdown expression of endogenous β-adducin in mouse keratinocytes.

2.3. Primary mouse keratinocyte culture and siRNA transfection

Newly isolated keratinocytes were grown as adherent cultures in a freshly prepared medium (365 ml DMEM medium, 2 mM glutamine, 100 unit/ml penicillin, 100 unit/ml streptomycin, 125 ml Hams F12 medium, 50 ml FBS, 2.5 mg transferrin, 2.5 mg insulin, 4.2 μg cholera toxin, 0.12 mg hydrocortisone, 17 mg adenine and 10 mg gentamicin) for one day and then cultured in KC-SFM medium containing low calcium (0.09 mM) (GIBCO, Australia) for five (D5) days. The D5 keratinocytes were treated with or without 1.4 mM Ca^{2+} for 24 h. The keratinocytes were then transfected either with control siRNA or β-adducin siRNA following the manufacturer's directions. The siRNA-transfected keratinocytes were recorded for cell morphology by an inverted light microscope at 45 h post-transfection. The cells were then either harvested for preparation of RNA and protein samples, or fixed in 4% formaldehyde in neutral buffer for immunofluorescence labeling.

2.4 Quantitative real-time reverse-transcription polymerase chain reaction

Total RNA was extracted from the siRNA-transfected keratinocytes using TRIzol reagent (Life Technologies, Australia) according to the manufacturer's instructions. RNA concentration, integrity and purity were analyzed using a spectrophotometer and agarose gel electrophoresis. Quality-checked 1 μg RNA per specimen was reverse transcribed using high capacity RNA-to-cDNA kit (LTC) in a final reaction volume of 10 μl containing random hexamer primers. The incubation conditions for reverse transcription (RT) were: 37 °C for 50 min, 95 °C for 10 min and hold at 4 °C indefinitely. A RT negative control (reactions with no reverse transcriptase) was prepared for testing DNA contamination.

The quantitative real-time PCR (qPCR) was performed to detect the expression of β-adducin, GAPDH and actin mRNAs using SYBR Green PCR master mix (LTC) in MicroAmp optical 384-well reaction plates (LTC) on an Applied Biosystems 7900HT Fast Real-Time PCR System. Two primers were designed for qPCR analysis of each gene, with β-adducin having a sense primer CCAAGACTTCAGAGGATACCAAGA and an anti-sense primer ATTCACCACCAGCCCTTCT; with GAPDH having the sense primer AACCTGCCAAGTATGATG and the anti-sense primer TCATTGTCATACCAGGAA and for actin having the sense primer CCTG GCTGGAGATAAGAATTACAT and the an anti-sense primer GTACTCGG CTTGGTCAGG. The primers were designed to span at least one exon–exon junction and to cover all the transcript variants found in Entrez Gene. Each qPCR reaction had a total volume of 10 μl that contained 13 ng cDNA templates, 200 nM forward primers, 200 nM reverse primers and 5 μl SYBR Green PCR master mixes. The thermal cycling conditions were: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 59 °C for 1 min, and a dissociation stage. Every combination of cDNA template and qPCR primer pairs was tested in triplicate. The threshold cycle (Ct) was determined by setting the threshold to 0.2, in the exponential phase of amplification, in the SDS 2.4 software (LTC). The qPCR efficiencies of all studied genes were similar and close to 100%, i.e. 1 thermal cycle (expressed as Ct) corresponds to a 2-fold change. Each qPCR product was verified by dissociation curve analysis and 2.2% agarose gel electrophoresis that showed single band with correct amplicon length and no primer dimmer. There was no positive amplification observed in the qPCR reactions using the RT negative control as template. The $2^{-\Delta\Delta Ct}$ method was used for the qPCR data analysis [22]. GAPDH was used as the reference genes for normalization.