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The Type III TGF- β receptor regulates filopodia formation via a Cdc42-mediated IRSP53: NWASP interaction in epithelial cells

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Synopsis

Cell adhesion and migration are tightly controlled by regulated changes in the actin cytoskeleton. Previously we reported that the TGF- β superfamily coreceptor, the type III TGF- β receptor (T β RIII/betaglycan), regulates cell adhesion, migration and invasion and suppresses cancer progression in part, through activation of the small GTPase, Cdc42, and Cdc42-dependent alterations to the actin cytoskeleton. Here we demonstrate that T β RIII specifically promotes filopodial formation and extension in MCF10A and HMEC mammary epithelial cells. Mechanistically, cell surface T β RIII and Cdc42 colocalize to filopodial structures and co-complex in a β -arrestin2 dependent, and a T β RI/T β RII independent manner. The β -arrestin2-mediated interaction between T β RIII and Cdc42 increases complex formation between the Cdc42 effectors, IRSp53 with N-WASP, to increase filopodial formation. We demonstrate a function link between filopodial structures and epithelial cell adhesion as regulated by the T β RIII-Cdc42 interaction. These studies identify T β RIII as a novel regulator of IRSP53/NWASP via Cdc42 to regulate filopodial formation and cell adhesion.

Keywords

T β RIII; filopodia; Cdc42; β -arrestin2; IRSp53

Introduction

Adhesion and migration of both tumor cells and host cells are essential components of the metastatic process. Both cell adhesion and migration are accompanied by changes in the actin cytoskeleton during epithelial to mesenchymal transition, intravasation and heterotypic cell interaction with blood cells and platelets during circulation, extravasation and colonization [1].

Filopodia are thin, actin-rich plasma-membrane protrusions that function as antennae for cells to probe their environment and have an important role in cell migration [2]. The Rho

(Ras homology) family small guanosine triphosphatase (GTPase) Cdc42 is a well-known regulator in the formation of filopodia [3]. Deregulation of Cdc42 has been demonstrated in several pathogenic processes including cancer, cardiovascular disease, and neuronal degenerative disease [4]. Several Cdc42 effectors N-WASP, IRSp53, PAK and MRCK have been implicated in filopodia formation. [5]. The Wiskott-Aldrich syndrome protein and neuronal-Wiskott-Aldrich syndrome protein (WASP/N-WASP) are scaffolds that link upstream signals to the activation of the ARP2/3 complex, leading to actin polymerization [6]. IRSp53 was identified as an effector for Rac1 and Cdc42, participating in filopodia and lamellipodia production. [7]. Recent studies have demonstrated that N-WASP is an essential mediator of IRSp53-induced filopodia formation. The SH3 domain of IRSp53 can bind N-WASP directly, with IRSp53 failing to induce filopodia in N-WASP KO fibroblast, while still maintaining the ability to induce lamellipodia formation and membrane ruffling [8]. IRSp53 can also generate filopodia by coupling membrane protrusion through its I-BAR domain with actin dynamics through SH4 domain binding partners including N-WASP and Mena [5]. Upstream mechanisms for regulating Cdc42:IRSp53: NWASP-mediated filopodial formations remain to be defined.

The ubiquitously expressed type III TGF- β receptor (T β RIII/betaglycan) is the most abundantly expressed TGF- β superfamily receptor [9]. T β RIII is classically thought to function as a coreceptor, presenting TGF- β superfamily ligands to their respective signaling receptors [10]. T β RIII mediates TGF- β superfamily ligand dependent as well as ligand independent signaling to both Smad and non-Smad signaling pathways [9]. T β RIII has essential roles in murine and chick development [11, 12], and has been defined as a suppressor of cancer progression/metastasis suppressor, with loss of T β RIII expression correlating with disease progression, advanced stage, and/or a poorer prognosis for patients [13–15].

T β RIII/betaglycan suppresses cancer progression primarily by regulating cancer cell motility and invasion [16–19]. Mechanistically, T β RIII suppresses motility of epithelial derived cancer cells via activation of Cdc42 in a β -arrestin2 dependent manner [18] and by regulating cell adhesion via integrin α 5 β 1 trafficking [20], which can act upstream of Cdc42 as well [21]. Here we examine the mechanism by which T β RIII regulates Cdc42 function in MCF10A and HMEC mammary epithelial cells.

Materials and Methods

Cell Culture and reagents

Immortalized but non-tumorigenic MCF10A and HMEC cells were cultured as described by ATCC or as previously described [22]. DMEM/F12 (1:1) media with 5% horse serum, EGF (epidermal growth factor, 20ng/ml), hydrocortisol (0.5 mg/ml), cholera toxin (100 ng/ml) and insulin (10 μ g/ml) for MCF10A and DMEM media with 10% fetal bovine serum (FBS) and insulin (10 μ g/ml) for HMEC's. COS7 and MEF cell lines were cultured in DMEM with 10% FBS. All cell lines were cultured at 37°C in a humidified incubator. Antibodies used were T β RIII (R&D System, Minneapolis, MN), Cdc42 (Cell Signaling Technology, Danvers, MA), Rac 1 (Santa Cruz Biotechnology, Santa Cruz, CA), β -arrestin 2 (Cell

Signaling Technology), IRSp53 (Santa Cruz Biotechnology) and NWASP (Cell Signaling Technology).

Transfection and Adenoviral Infection

Transient transfection was performed as described in transfection reagent manufacture's protocol. For T β RII and rat T β RIII transient transfection we used FuGENE (Roche Applied Science, Indianapolis, IN) and for siRNA to Control, Cdc42, Rac1 and IRSp53 (all from Santa Cruz Biotechnology) we used Lipofectamine (Invitrogen Life Technology, Carlsbad, CA). Cells were transfected for 48 hours for T β RII and 96 hours for shRNA and Control, Cdc42, Rac1 and IRSp53 before harvesting. Adenoviral infections were performed as previously described (2). For NTC and shT β RIII, adenoviral infections were done at multiplicity infection (MOI) of 50–75 in both MCF10A and HMEC and incubated for 96 hours before harvesting. For GFP-tagged CA/DN Cdc42 adenoviral infections were done at MOI of 100 and incubated for 36 hours before harvesting. shRNA to T β RIII was as described previously [20]. WASP constructs pRK5myc:WASP and pRK5myc:WASPH256 SH were kind gifts from Dr. Alan Hall.

Immunofluorescence

For actin staining, cells were plated at a low density to observe filopodia formation and serum depleted for indicated times. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X for 3 minutes. Blocking was performed with 1% BSA and then incubated with 1:50 dilution of phalloidin conjugated to Alexa Fluor 488 or Alexa Fluor 660 for 20 minutes. For T β RIII and Cdc42 staining, cells were fixed in 4% paraformaldehyde and permeabilized with 0.3% Triton X for 5 minutes, blocked with 1% BSA and then incubated with primary antibodies (antiCdc42 and anti-T β RIII, both 1:200 dilution) overnight. Secondary antibodies were used with donkey anti-goat conjugated to Alexa Fluor 555 and donkey anti-rabbit conjugated to Alexa Fluor 488 (Invitrogen Life Technology). Immunofluorescence images were obtained either using a Nikon inverted microscope or a Leica SP5 confocal microscope.

GST-PAK-CRIB pull-down assay and TGF- β Binding and Cross-Linking

GST-PAK-CRIB pull-down assay and TGF- β Binding and Cross-Linking were performed as previously described [13, 16, 18, 23] under the same conditions as for immunofluorescence.

Immunoprecipitation was performed essentially as described previously [20] and under the similar cell density and serum depletion conditions as described for immunofluorescence. Briefly cells were lysed in buffer containing 20mM Hepes pH7.4, 150mM NaCl, 2mM EDTA, 10% glycerol, 10mM NaF and 0.5% NP40 in the presence of protease inhibitors. Cells were lysed in buffer, incubated for 20 min on ice and then centrifuged at 15 000 g, 20 min at 4 °C. Equivalent amounts of protein were immunoprecipitated overnight with the antibody of interest and immune complexes were recovered on either protein G-Sepharose or protein A-Sepharose (GE Healthcare). Immunoprecipitates were washed four times with lysis buffer, twice with the same buffer without Tween-20, and then separated by SDS-PAGE. Proteins were then transferred to a Hybond-C extra nitrocellulose membrane (Amersham), probed with antibodies of interest, and detected by an enhanced

chemiluminescence technique. For IP with T β RIII, anti-T β RIII antibody was incubated with protein G-agarose beads. For IP with IRSp53 or NWASP, antibodies were incubated with protein A-agarose beads.

Western Blot Analysis

Membranes were incubated with primary antibodies overnight at 4 °C, washed, and incubated either with horseradish peroxidase-conjugated (1:2,000) or fluorescently tagged secondary antibodies (1:5,000) for 1 h at room temperature. Loading controls were performed using primary antibodies as indicated. Proteins were detected either by the ECL Plus chemiluminescence system (Amersham Biosciences) or the Odyssey infrared imaging system (Li-Cor Biotechnology, Lincoln, NE). Immunoreactive signals were captured on Hyperfilm ECL film and quantified by computer-assisted densitometry.

For **immunoprecipitation using binding and crosslinking followed by IP with GST-CRIB** beads, cells were first subjected to I-125 TGF- β binding crosslinking as described previously [13, 16, 18, 23]. Cells were then lysed in standard RIPA buffer with protease inhibitors and incubated with GST-PAK-CRIB beads. Immunoprecipitated samples were subject to SDS PAGE and exposed on phosphoimager to detect I-125 TGF- β bound, GST-CRIB immunoprecipitated receptors.

Live Cell imaging

MCF10A cells were infected with adenovirus expressing NTC or shT β RIII followed by transfection with 1 μ g of GFP tagged actin using Lipofectamine 48 hours prior to imaging. Cells were collected and replated in 35-mm glass bottom dishes (MatTek Corporation) in regular culture medium and placed in a temperature- and CO₂-controlled chamber. Zeiss Axio Observer Microscope equipped with 100x objective lens and 488nm filter was used for imaging. Images were taken every 15 seconds for 10 minutes with cooled charge-coupled device (CCD) video camera (Coolsnap ES high resolution CCD camera) operated by Metamorph image analysis software. Filopodia were counted manually for both live cell and fixed IF assays. For live cell analyses, NIH ImageJ's count function was used, starting at the top of the cell as if it were a clock and moving clockwise around the cell. Ten filopodia were measured for each of seven cells in each of the two conditions (shNTC and shT β RIII). For live-cell imaging, selected filopodia that persisted through at least 3 frames (not a focus error) were measured. The speed of extension started from the first frame (0 sec), and length of a given filopodium was measured at each 15 sec frame until maximal extension (the average was around 45 sec or 3 frames). A variety of relative filopodia sizes were selected within each condition, as well as a variety of the number of frames the filopodium persisted through, to minimize the influence of these factors. Speed of extension calculated by subtracting original filopodium length from final (fully extended) length and dividing by time to maximal extension. For fixed cell analyses all extensions were counted and number of cells for each condition is presented in the text.

Adhesion Assays were performed essentially as described previously [20]. Briefly, 96 well microtiter plates were pre-coated with 2.5 μ g/mL of fibronectin and saturated with 1% bovine serum albumin. Adherent cells were detached using 5 mM EDTA and resuspended at

a cell density of 25,000 cells/well (depending on the cell line) in serum free media for 60 minutes. Cells were washed in PBS and fixed in 4% paraformaldehyde and then stained with crystal violet for 20 min. The stain was washed, dried and color solubilized using 2% SDS for 20 min. Absorbance was read at 595 nm on the Wallac Victor Counter (PerkinElmer Life Sciences) and values normalized to BSA only controls

Statistical analysis

Significance of results was assessed using either standard student t-test, or ANOVA analyses followed by post hoc analysis of either a 1 sample t-test to compare the experimental condition to the control or a 2 –tailed unpaired Student's *t* test, with a minimum of a 95% confidence interval. These are indicated in legends.

Results

TβRIII promotes filopodia formation and extension

We have previously demonstrated that restoring TβRIII expression can regulate cell adhesion and migration via effects on the actin cytoskeleton [18, 20]. To determine the mechanism by which TβRIII regulates the actin cytoskeleton in epithelial cells, we silenced endogenous TβRIII expression in the immortalized, but non-tumorigenic MCF10A and HMEC human mammary epithelial cells, and examined effects on serum starvation induced filopodia formation. shRNA-mediated silencing of TβRIII expression (shTβRIII) in MCF10A and HMEC cells (Fig. 1A and Supp. Fig. 1) significantly decreased both the number (Fig. 1C, Supp. Fig. 1) and length (Fig. 1E) of the filopodia relative to non-targeting shRNA control (NTC) (Fig. 1B). The effect of shTβRIII was specific as we were able to rescue the effects of shRNA-mediated silencing of TβRIII expression on filopodia with shTβRIII-resistant rat TβRIII (Fig. 1C, Supp. Fig. 1), which effectively restored TβRIII expression (Fig. 2A).

Filopodia grow and retract after their initiation, exhibiting rich dynamical behaviors. To investigate the mechanism by which TβRIII was regulating filopodial number and length we used sequential time-lapse wide field fluorescence microscopy on control MCF10A-shNTC and MCF10A-shTβRIII cells expressing actin–GFP to follow dynamics of the actin cytoskeleton as described in detail in Methods. The live cell analyses (Fig. 1D–F) focused on cytoskeletal structures in dynamic flux between microspikes and filopodia, with the majority of the structures returning to a short microspike-like state. Filopodia selected for quantification were cycling through elongation and retraction, commonly observed for filopodial structures. We find that shRNA-mediated silencing of TβRIII expression decreased the rate of filopodial extension (Fig. 1F), suggesting that TβRIII regulates filopodial number and length through regulation of the rate of filopodial extensions.

TβRIII is required for maximal activation of Cdc42 in human mammary epithelial cells to regulate filopodia formation

The small Rho-subfamily GTPase, Cdc42, is a well-established regulator of filopodia formation. We have previously demonstrated that restoring TβRIII expression in cancer cells constitutively activated Cdc42 and Rac1 [18]. Accordingly, we investigated the effects of

silencing endogenous T β RIII expression on Cdc42 and Rac1 activity in human mammary epithelial cell lines under conditions that stimulated filopodia formation (serum starvation). In both MCF10A and HMEC human mammary epithelial cell lines, shRNA-mediated silencing using two independent shRNA's to T β RIII decreased Cdc42 activation (Fig. 2A, Supp. Fig. 2), and this effect was specific as the shRNA-mediated silencing decrease in Cdc42 activation could be rescued with shRNA resistant rat T β RIII (Fig. 2A). Interestingly, rescue of T β RIII expression in shT β RIII cells did not further enhance CDC42 activation in part due to high basal Cdc42 activation under serum-starved conditions in shNTC cells. In contrast, shRNA-mediated silencing of T β RIII had no effect on Rac1 activation in either MCF10A or HMEC cells (Fig. 2B) consistent with our prior observation on T β RIII's effects on Rac1 in cancer cells [18]. These support a specific effect of T β RIII on Cdc42 in human mammary epithelial cells and indicate that while T β RIII is required for maximal activation, it may not further enhance activation of cells that have constitutively high active Cdc42 levels as observed for the MEC's under serum free conditions (Fig. 2A, shNTC)

To investigate whether Cdc42 was downstream of T β RIII in regulating filopodia formation, we expressed either dominant negative Cdc42 (DN-Cdc42) or constitutively active Cdc42 (CA-Cdc42) in the presence or absence (shT β RIII) of T β RIII expression (Fig. 2C). Similar to the effect of shRNA-mediated silencing of T β RIII expression, DN-Cdc42 decreased filopodial number to the same level as shT β RIII (Fig. 2C,D). Importantly, in the presence of DN-Cdc42, shRNA-mediated silencing of T β RIII expression was unable to further decrease filopodial formation, suggesting that T β RIII was functioning through Cdc42 to mediate its cellular effects (Fig. 2C,D). Consistent with a role for Cdc42 downstream of T β RIII, CA-Cdc42 was able to rescue 50% of the phenotype of shRNA-mediated silencing of T β RIII expression on filopodial formation (Fig. 2C, D), without altering basal filopodial formation. Taken together, these data indicate that T β RIII regulates filopodia formation at least in part through activation of Cdc42 in human mammary epithelial cells.

T β RIII co-complexes specifically with Cdc42 and co-localize to the cell edge and filopodial structures

The localization of small Rho-GTPases is thought to have an important role in regulating their function. As T β RIII activates Cdc42 to regulate filopodia formation, we first examined the localization of endogenous T β RIII upon stimulation of filopodia in MCF10A cells using serum starvation. Endogenous T β RIII localized in a punctate fashion to filopodial structures and at the base of the filopodial structures (Fig. 3A). Upon examining the spatial relationship between endogenous Cdc42, T β RIII and the actin cytoskeleton in mammary epithelial cells, endogenous T β RIII localized along the cell membrane, and along the lengths of filopodia (Fig. 3A, B), while endogenous Cdc42 localized along the cell membrane and on filopodial structures (Fig. 3B). Perinuclear localization for T β RIII, as have been noted for other internalized receptors [24] and nuclear localization for Cdc42 was also observed. Endogenous Cdc42 and T β RIII co-localized at the cell membrane and along filopodial extensions (Fig. 3B). To determine whether T β RIII and Cdc42 existed in a complex, we performed co-immunoprecipitation analysis of the endogenous proteins. In both MCF10A and HMEC cells, immunoprecipitating endogenous T β RIII resulted in the co-immunoprecipitation of endogenous Cdc42 (Fig. 3C). shRNA-mediated silencing of

endogenous T β RIII expression decreased the amount of Cdc42 immunoprecipitated by T β RIII antibody (Fig. 3C), supporting the specificity of the interaction.

To investigate whether cell surface T β RIII formed a complex specifically with activated Cdc42, we utilized two distinct approaches. First, we examined the interaction between T β RIII and WASP, which binds with much higher affinity to Cdc42 than Rac1 [25] and depends on the nucleotide state of Cdc42 [26]. We find that endogenous T β RIII co-immunoprecipitated with WASP (Fig. 4A) in MCF10A cells. WASP interacts with Cdc42 via amino acids 201–321 [25]. To determine if the interaction was dependent on the ability of WASP to interact with Cdc42-GTP, we utilized the WASP mutant WASP H246 SH [25] and find that it was deficient at co-complexing with endogenous T β RIII (Fig. 4A). As an alternate method to examine T β RIII's ability to interact with Cdc42 in its GTP bound state, we labeled cell surface TGF β receptors with 125 I-TGF β ₁, and performed a pulldown with GST beads conjugated to PAK-CRIB, which binds activated Cdc42 and Rac1 [27], or GST alone as control. GST-PAK-CRIB was able to pull down cell surface T β RIII, along with T β RII and T β RI in both MCF10A and HMEC cells (Fig. 4B), suggesting that perhaps, via T β RIII, all three TGF β receptors may interact with activated Cdc42/Rac1 (Fig. 4B,C), consistent with what has been reported for T β RII and T β RI previously [23]. Consistent with this hypothesis, the interaction of T β RIII and T β RII with activated Cdc42/Rac1 was decreased (Fig. 4B) by shRNA-mediated silencing of T β RIII expression (Fig. 4C), demonstrating the specificity of the T β RIII–Cdc42 interaction and suggesting that T β RIII may be required for the T β RII/T β RI–Cdc42/Rac1 interaction as well. Conversely, increasing T β RIII expression at the cell surface (Fig. 4C) in both MCF10A and HMEC cells resulted in increase in the amount of T β RIII that interacted with Cdc42.

As GST-PAK-CRIB beads interact with activated Cdc42 and Rac1, to investigate the specificity of the interaction with cell surface TGF β receptors, we individually silenced Cdc42 and Rac1 expression using siRNA, and performed GST-PAK-CRIB pulldown assays. While silencing of endogenous Cdc42 expression decreased GST-PAK-CRIB-mediated immunoprecipitation of cell surface T β RIII, silencing of endogenous Rac1 had no effect (Fig. 4D), supporting a specific interaction between cell surface T β RIII and activated Cdc42.

Taken together, these data indicate specific co-complex formation between T β RIII and Cdc42-GTP.

T β RIII can interact with activated Cdc42 independent of T β RII and T β RI

Although T β RIII functions as a co-receptor for TGF β receptors, including T β RII and T β RI, T β RIII is present in abundance relative to T β RII and T β RI. Accordingly, we investigated whether the interaction of T β RIII with activated Cdc42 depends on T β RII or T β RI using a series of mouse lung epithelial cell lines, wild type MV1Lu, which express all three TGF- β receptors, R1b, which lacks T β RI expression and DR, which lack T β RII and T β RI expression [28]. GST-PAK-CRIB pulled down cell surface iodinated T β RIII, along with T β RII and T β RI in wild type MV1Lu, T β RIII and T β RII in R1b cells and T β RIII in DR cells (Fig. 5A). DR cells with GST alone beads did not pull down any T β RIII (Fig. 5A). While the extent of the interaction with T β RIII was modestly diminished in DR cells (Fig. 5A),

suggesting that T β RII might contribute to the interaction, T β RIII was still able to interact with activated Cdc42 in the absence of T β RII and T β RI.

T β RIII forms a complex with T β RII, which subsequently phosphorylates T β RIII on its short cytoplasmic domain [29] and can also regulate signaling of other TGF- β superfamily ligands including activin and BMP, via their type II receptors [30]. To investigate the contribution of the kinase activity of T β RII, and other TGF- β superfamily type II receptors, ActRII and BMPRII, to T β RIII's interaction with Cdc42, we expressed exogenous T β RIII with the wild type type II receptors and/or kinase dead T β RII, which functions as a dominant negative, in COS7 cells, labeled the cell surface receptors with I¹²⁵TGF β ₁ and pulled down labeled receptors with GST-PAK-CRIB beads. Consistent with our prior results, both endogenous T β RIII, which is present at low but detectable levels, and T β RII, and exogenously expressed T β RIII and T β RII were pulled down by GST-PAK-CRIB (Fig. 5B). Expression of kinase dead T β RII reduced the level of binding of T β RIII and T β RII to GST-PAK-CRIB (Fig. 5B). Increasing expression of ActRII and BMPRII had no effect on altering T β RIII's interaction with GST-PAK-CRIB (Supp. Fig. 8). These data indicate that while T β RIII can interact with activated Cdc42 independently of the type II receptors T β RII, ActRII and BMPRII or T β RI, the kinase activity of T β RII enhances T β RIII's interaction with activated Cdc42.

β -arrestin2 is essential for T β RIII's interaction with GTP-Cdc42

To determine the structural determinants on T β RIII required for the interaction between T β RIII and Cdc42, we overexpressed either GFP control, T β RIII (wild type full-length T β RIII) or T β RIII Cyto (T β RIII lacking cytoplasmic domain) in MCF10A cells. Using GST-PAK-CRIB pulldown we determined that, interaction between GST-PAK-CRIB and GFP expressing cells was below detectable levels. However increasing T β RIII levels resulted in co-complex formation between T β RIII and GST-PAK-CRIB (Fig. 6A, top). In contrast, T β RIII Cyto was pulled down much less efficiently, with a 60% decrease relative to T β RIII (Fig. 6A, B), suggesting that the cytoplasmic domain of T β RIII mediated the interaction of T β RIII with active Cdc42. Aside from interaction with and phosphorylation by T β RII, the cytoplasmic domain of T β RIII has been demonstrated to interact with two scaffolding proteins, β -arrestin2, via threonine 841, and GIPC, via a class I PDZ binding motif comprised of the last three amino acids of T β RIII. To examine the contribution of T β RIII's interaction with β -arrestin2 and GIPC, we utilized T β RIII-T841A (β -arrestin2 binding deficient) and T β RIII-DEL, (GIPC binding deficient) [31]. While T β RIII-DEL was pulled down by GTP bound Cdc42 (Fig. 6A,B) T β RIII-T841A was pulled down much less efficiently, with a 70% decrease relative to T β RIII (Fig. 6A, B), suggesting that the interaction of T β RIII with Cdc42 was mediated by the interaction of T β RIII with β -arrestin2.

To investigate the role of β -arrestin2 in the interaction of T β RIII and activated Cdc42, we utilized two distinct approaches. First, we used siRNA to β -arrestin2 in MCF10A cells. Compared to siRNA control cells, the interaction of cell surface T β RIII with activated Cdc42 was significantly reduced in si- β -arr2 MCF10A cells (Fig. 6C). As an alternative approach, we used mouse embryonic fibroblasts (MEF's) derived from β -arrestin2 knock out mice [32]. Compared to MEFs obtained from wild type (WT) littermate controls, the interaction of cell surface T β RIII with activated Cdc42 was significantly reduced in β -

arrestin2 $-/-$ MEFs (Fig. 6D). In addition, activation levels of active Cdc42 were also reduced in β -arrestin2 $-/-$ MEF cells compared to β -arrestin2 $+/+$ cells (Supp. Fig. 4), consistent with our previously published observations demonstrating a role for β -arrestin2 in Cdc42 activation (Myhre and Blobel 2009). Taken together, these data suggest that the interaction of T β RIII with Cdc42 is mediated via β -arrestin2 and is required for efficient Cdc42 activation.

To investigate the role of the interaction of T β RIII with Cdc42 via β -arrestin2 on filopodial formation, we transiently knocked down T β RIII expression and rescued with shRNA resistant rat wild type T β RIII, T β RIII Cyto, T β RIII-DEL and T β RIII-T845A (Supp. Fig. 5). Consistent with our prior results, shRNA-mediated silencing of T β RIII expression decreased filopodial formation (Fig. 6E, F), which was rescued by wild type T β RIII. In addition, T β RIII-DEL was able to effectively rescue filopodial formation, while T β RIII Cyto and T β RIII-T845A were unable to do so (Fig. 6E, F). These data strongly support a role for T β RIII via β -arrestin2 interacting with Cdc42 in regulating filopodial formation in human mammary epithelial cells.

T β RIII regulates IRSp53 and NWASP interaction via Cdc42 activation to regulate cell adhesion

The Rho GTPase, Cdc42, has a number of downstream effectors. One of these effectors, IRSp53 has a specific role in mediating filopodial formation in a context dependent manner [7, 33]. In addition, increasing IRSp53 in most cell types induces extensive actin-containing cell protrusions that are dependent on Cdc42 and Rac1 [7]. IRSp53 has been demonstrated to directly interact with N-WASP as well, with this interaction being required for IRSp53-induced filopodial formation [5]. To investigate whether IRSp53 has a role in filopodial formation in human mammary epithelial cells, we silenced IRSp53 expression and examined the actin cytoskeleton of MCF10A cells. Compared to the control, siRNA-mediated silencing of IRSp53 expression decreased filopodial formation in MCF10A cells (Fig. 7A–C). As we have established a role for Cdc42 in regulating filopodial formation in human mammary epithelial cells, we investigated whether altering Cdc42 activation was able to alter the interaction of IRSp53 and NWASP in mammary epithelial cells. Constitutively activate Cdc42 (CACdc42) increased the interaction of IRSp53 and NWASP (Fig. 7D, Supp. Fig. 6A), while dominant negative Cdc42 (DNCdc42) decreased the interaction of IRSp53 and NWASP compared to GFP control cells (Fig. 7D, Supp. Fig. 6A). To determine the functional link between T β RIII- β -arrestin2 and the IRSp53 and N-WASP complex that regulates filopodial formation, we determined whether T β RIII was required for the IRSp53-NWASP interaction. We silenced T β RIII expression, rescued with shRNA resistant wild type T β RIII or T β RIII-T845A, and assessed the interaction of IRSp53 and N-WASP in MCF10A cells. Consistent with a role for T β RIII, shRNA-mediated silencing of T β RIII expression decreased the interaction of IRSp53 with N-WASP (Fig. 7E, Supp. Fig. 6B). Importantly, the effect of shRNA-mediated silencing of T β RIII expression on the interaction of IRSp53 with N-WASP could be rescued by wild type T β RIII but not by T β RIII-T845A (Fig. 7E, Supp. Fig. 6B). These data demonstrate that the T β RIII- β -arrestin2 interaction is important for filopodia formation in human mammary epithelial cells, with T β RIII

mediating the interaction of IRSp53 and NWASP in a β -arrestin2 and Cdc42 dependent manner.

We have recently demonstrated a role for T β RIII in regulating epithelial cell adhesion that depends on β -arrestin2 [20]. In addition, IRSp53 has recently been shown to regulate cell-ECM adhesion signaling in epithelial cells [34]. Therefore, to examine the functional link between filopodia formation and cell adhesion in mammary epithelial cells, we silenced IRSp53 expression and examined the effect on adhesion to fibronectin (FN) in MCF10A cells. Compared to the control, siRNA-mediated silencing of IRSp53 expression decreased cell adhesion in MCF10A cells (Fig. 7F). To further determine if T β RIII effects cell adhesion via Cdc42, which in turn regulates the IRSp53-NWASP interaction, we examined the effect of altering CDC42 upon altering T β RIII expression. As we previously reported [20], shT β RIII significantly reduced cell adhesion to FN, while increasing T β RIII expression significantly enhanced cell adhesion (Fig. 7G, Supp. Fig. 7). Consistent with a role for Cdc42 downstream of T β RIII, CA-Cdc42 was able to rescue the adhesion defect in shT β RIII cells (Fig. 7G, Supp. Fig. 7). Conversely, DNCdc42 was able to significantly diminish T β RIII induced cell adhesion (Fig. 7G). These data demonstrate a functional link between the T β RIII-Cdc42 complex in downstream IRSp53-NWASP dependent filopodia formation and cell adhesion in mammary epithelial cells.

Discussion

The ability of T β RIII to suppress cancer progression is due, in part, to inhibition of migration and invasion in cancer cells [9]. We have reported specific effects of T β RIII on directional migration of epithelial-derived cancer cells in a Cdc42 dependent manner [18] and effects of T β RIII on cell adhesion via integrin α 5 β 1 [20], which can function upstream of Cdc42 [21]. Here, we demonstrate that in mammary epithelial cells T β RIII is required for filopodial formation, via direct co-complexing with active Cdc42 to regulate Cdc42 activation. Further, we demonstrate an essential role for IRSp53 in filopodial structure organization to regulate cell adhesion in epithelial cells and find that T β RIII is required for the interaction of IRSp53 and NWASP to regulate filopodia formation downstream of Cdc42.

The mechanism by which Cdc42 specifically promotes filopodia formation is controversial and an active area of investigation. Over 30 target proteins have been identified that interact with either or both Cdc42 and Rac1 [35] [7]. Several studies implicate a WASP/NWASP interaction with Arp2/3 downstream of Cdc42 in filopodia formation *in vitro* [36] and *in vivo* [37, 38]. IRSp53, which is a target of Cdc42 [33], has been shown to bind Mena and Ena/VASP family proteins to promote filopodia formation [7]. IRSp53 has also been shown to be a downstream effector of Rac1 [37]. Here we tested the interactions of NWASP-ARP2/3 and IRSp53-Mena in the presence and absence of T β RIII in mammary epithelial cells but were unable to detect interaction of the endogenous proteins (data not shown). Thus the mechanisms by which Cdc42 regulates filopodia formation are likely to be context dependent.

Cdc42 regulates multiple cellular functions including motility, proliferation, apoptosis and cell morphology. To perform these diverse roles, the timing and location of Cdc42 activation must be tightly controlled [39]. Spatiotemporal dynamic studies of Cdc42 localization revealed localized activation of Cdc42 in the cell periphery at cell edges extending filopodia but not within the filopodia themselves [39]. However, EGF stimulated filopodia have been shown to contain active Cdc42 [40]. IRSp53 has been localized to the very edge or tips of filopodia [41] and is activated by an interaction with Cdc42. NWASP has also been found to be active on extending filopodia [40], implying the presence of activated Cdc42 in filopodial structures. We observe Cdc42 and T β RIII colocalization both at the cell periphery and in extended filopodia, and demonstrate that the T β RIII–Cdc42 interaction is essential for Cdc42 activation and filopodial formation in epithelial cells. Our data suggest that T β RIII activates Cdc42 by interactions on the cell periphery and possibly in the filopodia as well. Our studies also demonstrate specifically the ability of cell surface T β RIII to co-complex with active Cdc42. Further studies will focus on the dynamics of the interaction in a spatio-temporal fashion.

We also observed that while the cytoplasmic domain of T β RIII significantly diminished co-complex formation between T β RIII and GTP-Cdc42, it was not completely abrogated. We hypothesize that the transmembrane domain of T β RIII, which can interact with T β R II or T β RI, may contribute to the interaction, as T β R II and T β RI can also interact with Cdc42 [23].

TGF- β binds either to T β RIII which presents the ligand to dimeric T β R II or directly to T β R II. Ligand binding to T β R II leads to recruitment of T β RI and activates T β RI kinase activity which can initiate Smad and non-Smad signaling pathways [42]. TGF- β induces actin cytoskeleton mobilization in cells, and these effects appear to be dependent upon non-Smad signaling [43, 44]. TGF- β can also activate Cdc42 [43] [18]. Here we demonstrate a specific interaction between active Cdc42 and T β RIII/T β R II/T β RI. Interestingly, T β RIII's interaction with active Cdc42 is only partially dependent on the presence of T β R II or T β RI (Fig. 5A, B). Interactions of T β R II and T β RI with Cdc42 have been reported [23]. Interestingly, we find in both MCF10A and HMEC cells, loss of T β RIII resulted in a reduction in the binding of T β R II to active Cdc42 (Fig. 4) indicating a requirement for T β RIII in forming a functional complex between the TGF- β receptors and Cdc42. In addition, reduction of T β RI binding in shT β RIII cells was also observed (Fig. 4B), which could either be due to reduced ligand presentation in the absence of T β RIII or due to decreased T β RIII-mediated TGF- β receptor complex formation with Cdc42-GTP. We did observe some variability in total Cdc42 levels upon increasing T β RIII expression, but this was not consistent. Conversely, as T β RIII can interact with Cdc42GTP in the absence of T β RI (Fig. 5A, 5B) overexpression of T β RIII may sequester Cdc42-GTP reducing the amount of Cdc42-GTP available to complex with T β RI (Fig. 4B, C). The role of activated Cdc42/Rac1 in interacting with distinct TGF- β receptors remains to be explored.

Efforts to define the mechanisms mediating T β RIII's roles in cell migration and invasion to regulate cancer progression have revealed several mechanisms including T β RIII dependent down regulation of NF κ B via β -arrestin2 and activation of Cdc42 via β -arrestin2 and regulation of integrin trafficking also via β -arrestin2 [20] [18, 45]. These studies have

defined β -arrestin2 as a key regulator for facilitating these interactions. T β RIII–Cdc42 interaction via β -arrestin2 may be required for proper transport of Cdc42 to sites of actin nucleation. As such, we have recently demonstrated that T β RIII can regulate integrin α 5 β 1 trafficking via β -arrestin2 [20] and hence similar trafficking consequences might be observed in the context of Cdc42 as well. Here we demonstrate a novel role for T β RIII in regulating filopodial formation via activation of Cdc42 and effects on the downstream Cdc42 effectors, IRSp53 and NWASP. The activation of Cdc42 in response to upstream signaling is regulated by guanine nucleotide exchange factors (GEFs) [46, 47] and GTPase activating proteins. Current studies are directed at identifying the GEF's specifically coupling the TGF- β receptors to Cdc42 to regulate epithelial cell filopodial formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. TβRIII promotes filopodial formation and extension in human mammary epithelial cells

(A) MCF10A mammary epithelial cells adenovirally infected with either shRNA to TβRIII (shTβRIII) or non-targeted control shRNA (shNTC) were harvested 96 hours after infection and TβRIII expression levels assessed either by I-125 TGF-β binding and crosslinking followed by immunoprecipitation for TβRIII to examine cell surface levels (upper panel) or by western blot in the total cell lysates. β-actin was used as a loading control. (B) MCF10A cells either shNTC or shTβRIII, or shTβRIII followed by rescue with rat TβRIII (rTβRIII) cells were serum starved to induce filopodia, fixed and stained for actin using phalloidin-conjugated to Alexa 488. Only adenovirus expressing cells were examined. Representative

examples of the spectrum observed are presented with the number in each panel indicating the number of filopodia for the presented cell (C) Average filopodial number per cell was quantitated (Methods), and presented. Data represent the mean $\pm$ SE of three independent experiments with >150 cells per condition counted. *, $p < 0.005$, **, $p < 0.05$ (D–F) Filopodial dynamics of shNTC and shT β RIII MCF10A cells. Cells were imaged using actin-GFP for 10 minutes as described in Methods, with images acquired every 15 seconds, Bar= 1 μ m. Average length (E) and extension speed (F) of 40 filopodia/condition were quantified and presented here. Arrows indicate filopodia. **, $p < 0.05$.

Figure 2. TβRIII activates Cdc42 in human mammary epithelial cells

(A, B) MCF10A (left) and HMEC (right) cells infected with shNTC, shTβRIII, and shTβRIII followed by rescue with rat TβRIII (rTβRIII), were assayed after serum starvation to mimic filopodia-inducing conditions for either Cdc42-GTP (A) or Rac1-GTP levels (B) as described in Methods. 5% of total cell lysate was used for input. Representative experiment of 3 replicates is presented. (C–D) MCF10A were infected with shNTC or shTβRIII, followed by infection with adenovirus expressing GFP (control), GFP tagged constitutively active Cdc42 (CACdc42) or dominant-negative Cdc42 (DNCdc42), serum starved for 14–16 hours then fixed and stained for actin using with phalloidin conjugated Alexa Fluor 660. Cells with both dsRed (to select cells expressing shNTC or shTβRIII) and GFP (to detect cells expressing CACdc42 or DNCdc42) were examined using confocal microscopy and counted for filopodia. Examples of cells under each condition are presented with the number in each panel indicating the number of filopodia for the presented cell. Average number of filopodia per cell of 3 independent experiments is shown. * $p < 0.05$

Figure 3. Cell surface TβRIII interacts with and colocalizes with Cdc42 at the cell edge and on filopodial structures

Confocal images of sub-confluent MCF10A cells serum starved for 14 hours before fixation and staining for (A) endogenous TβRIII (green) or actin (red) using phalloidin. Arrows indicate representative points of punctate localization of TβRIII at filopodial structures and at the base of filopodia. Bar=10μm or (B) endogenous TβRIII (red) and endogenous Cdc42 (green) were labeled using antibody to TβRIII and Cdc42. Closed arrows indicate representative areas where TβRIII and Cdc42 colocalize. Open arrows in magnified areas of boxed regions indicate areas where TβRIII and Cdc42 colocalize. White Bar= 3.3 μm, Black Bar=2.2μm. (C) Indicated MCF10A cells were immunoprecipitated with antibody to TβRIII and immunoblotted with antibody to endogenous Cdc42, with assessment of <5% of the cell lysate for TβRIII and Cdc42. Data are representative of >3 independent experiments. Quantitation relative to total input Cdc42 is presented below the immunoblot.

Figure 4.

(A) MCF10A cells transiently transfected with either wild type WASP construct or WaspH246 SH were immunoprecipitated with anti-Myc antibody and immunoblotted for TβRIII or immunoprecipitated with TβRIII and immunoblotted for Myc as indicated. 5% of cell lysate was used to detect total TβRIII and expression of WASP constructs. Integrated density of the amount of TβRIII and MYC immunoprecipitated relative to inputs is presented using Odyssey infrared imaging system (LI-COR, Lincoln, NE) (B) MCF10A and HMEC cells expressing either shNTC, shTβRIII or full length TβRIII (FLTβRIII), were serum starved for 14 hours and subjected to 125 I-TGF- β_1 binding and cross-linking followed

by pulldown (PD) with GST-CRIB beads (Methods) for active Cdc42. Parallel GST-PAK CRIB pull downs were performed to determine active Cdc42 (GTP-Cdc42) levels as well. A representative experiment of 2 replicates of each cell line is presented with β -actin and Cdc42 as a loading control. For quantification of signal, Odyssey infrared imaging system (LI-COR, Lincoln, NE) was used and the quantification for the fold changes relative to total Cdc42 levels is presented (C) Parallel ^{125}I -TGF- β_1 binding and crosslinking studies of total cell lysates were performed to observe knockdown and overexpression of T β RIII at the cell surface. (D) MCF10A cells were subjected to siRNA mediated silencing with either non targeting control oligos (siCtl), Cdc42 (siCdc42) or Rac1 (siRac1). After 14 hours of serum starvation, cells were subjected to ^{125}I -TGF- β_1 binding and cross-linking followed by pulldown with GST-CRIB beads for active Cdc42 or Rac1. Immunoblots examining extent of Cdc42 and Rac1 knockdown and levels of T β RIII are presented below. A representative experiment of 3 replicates is presented. Graph of fold average T β RIII intensity relative to control of 3 independent experiments is presented (Right). * $p < 0.01$

Figure 5. Surface TβRIII interacts with GTP bound Cdc42 in a TβRII and TβRI independent manner

(A) Mink lung epithelial cell lines MV1Lu (wild type), R1b (TβRI^{-/-}) and DR (TβRI^{-/-} and TβRII^{-/-}) were serum starved for 14 hours and subjected to ¹²⁵I-TGF-β₁ binding and cross-linking followed by GST-CRIB pulldown assay (PD) with GST only as a control. Expression level of TβRI, TβRII and TβRIII of MV1Lu, R1b and DR cells were detected by ¹²⁵I-TGF-β₁ binding and cross-linking (right panel). A representative of two independent replicates is presented. β-actin was used as a loading control. Integrated density, normalized to total TβRIII, is presented below and fold integrated density of TβRIII for each cell line relative to MV1Lu cells is presented below. (B) COS7 cells infected with adenovirus expressing either GFP or TβRIII followed by transfection with empty vector, TβRII-HA or kinase dead TβRII (TβRIIKD-HA) were subjected to ¹²⁵I-TGF-β₁ binding and cross-linking followed by GST-CRIB pulldown assay. 5% of total cell lysate was used for input to immunoblot against TβRIII, β-actin and anti-TβRII using Odyssey infrared imaging system to determine expression of transfected receptors. Control TβRIII intensity was subtracted

from each condition, normalized to total T β R_{III} and fold T β R_{III} intensity bound to GST-CRIB relative to T β R_{III} only condition is presented. Quantified data represent the average of five independent experiments. ANOVA; $p < 0.0001$; one sample t-test and two sample t-test: $*p < 0.005$

Figure 6. β -arrestin2 is required for T β RIII's interaction with and activation of Cdc42
 (A–B) MCF10A cells infected with adenovirus expressing GFP (control), T β RIII (wild type full length T β RIII), T β RIII Cyto (T β RIII lacking cytoplasmic domain), T β RIII-Del (cannot bind to GIPC) and T β RIII-T841A (cannot bind to β -arrestin2) were serum starved for 14 hours and subjected to pulldown (PD) with GST-PAK-CRIB beads. Pulldowns were subject to immunoblotting against T β RIII. 5% of lysate was used as inputs and immunoblotted against T β RIII, Cdc42 and β -actin using Odyssey infrared imaging system. (B) T β RIII pulled down with GST-PAK CRIB for each condition is normalized to total T β RIII for each condition then fold relative to T β RIII is presented. For quantification of signal, Odyssey infrared imaging system (LI-COR, Lincoln, NE) was used and the quantification for the fold changes for four independent experiments is presented. ANOVA; $p < 0.005$; one sample t-test $*p < 0.05$ C) MCF10A cells were subjected to siRNA mediated silencing with either non targeting control oligos (siCtl), or β -arrestin2 (si β -arr2). After 14 hours of serum starvation, cells were subjected to 125 I-TGF- β_1 binding and cross-linking followed by pulldown with GST-CRIB beads for active Cdc42 or Rac1. Top panel represents cell surface T β RIII in siCtl and si β -arr2 cell interacting with GST-PAK-CRIB. As input T β RIII, β -arrestin2 and β -actin levels are presented (D) Mouse embryonic fibroblasts (MEF cell lines), wild type (β -arr2 +/+) and β -arrestin2 knock out (β -arr2 -/-) were subjected to 125 I-TGF- β_1 binding and cross-linking and then to GST-PAK-CRIB pulldown assays. Top panel represents cell surface T β RIII in WT and β -arr2 KO MEF pulled down with GST-PAK-CRIB. As input T β RIII β -arrestin2 and β -actin are shown. Data represent one of three independent

experiments. (E–F) MCF10A cells infected with adenovirus expressing shNTC or shT β RIII, were transfected with control empty vector (EV), rat T β RIII (rT β RIII, column 3), rat T β RIII cyto (rT β RIII cyto), rat T β RIIIDel (rT β RIIIDel) and rat T β RIIIT845A (rT β RIIIT845A, T β RIII mutant which cannot bind to β -arrestin2, column 6). After 48 hours, cells were re-plated on the coverslips and serum starved for 14 hours. Cells were fixed and actin stained with phalloidin. Arrows indicate filopodia (E). (F) Filopodia in each condition (n>50) were counted as described in Methods. Data represent the average of three independent experiments. ANOVA test was used to determine difference between groups followed by post hoc t- tests. * 1 sample t- test, ** standard 2 tailed t-test. Both * and ** have p<0.05.

Figure 7. TβRIII enhances IRSp53 and NWASP interaction to regulate filopodia formation and cell adhesion in epithelial cells

(A–C) MCF10A cells transfected with either control siRNA (siCtrl) or siRNA to IRSp53 (siIRSp53) were either (A) immunoblotted against IRSp53 to determine extent of knockdown (B) fixed and stained for actin with phalloidin conjugated with Alexa Fluor 488. (C) >20 cells/condition in two experiments were counted for filopodia and average number of filopodia is compared in both conditions. *, $p < 0.01$. (D) MCF10A cells infected with either control GFP adenovirus (control), constitutively active Cdc42 (CACdc42) or dominant-negative Cdc42 (DNCdc42) and subjected to anti-IRSp53 immunoprecipitation (IP) and probed for NWASP. NWASP, IRSp53 and TβRIII are shown as input controls. Integrated density of immunoprecipitated WASP, normalized to total WASP, is presented below (E) shNTC or shTβRIII MCF10A cells transfected with empty vector, rTβRIII or rTβRIII845A were immunoprecipitated-using antibody to endogenous IRSp53 and immunoblotted against endogenous NWASP. Inputs were probed for IRSp53, NWASP and TβRIII as input controls. Integrated density relative to input WASP is presented. For quantification of signal of D and E, Odyssey infrared imaging system (LI-COR, Lincoln, NE) was used. (F–G) MCF10A cells transfected with (F) either control siRNA (siCtrl) or siRNA to IRSp53 (siIRSp53) or (G) MCF10A cells with shNTC and shTβRIII transfected with empty vector (EV), rTβRIII or rTβRIII845A, overexpressing TβRIII (TβRIII-HA) alone or with DNCdc42 and CACdc42 as indicated were plated on fibronectin coated 96 wells for 1 hr, and adhesion levels determined as described in Methods. ANOVA test was

used to determine difference between groups followed by post hoc unpaired 2 tailed student t- tests between indicated groups. *, $p < 0.05$