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The Rho Target PRK2 Regulates Apical Junction Formation in Human Bronchial Epithelial Cells[▽]

Sean W. Wallace, Ana Magalhaes,‡ and Alan Hall*

Cell Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10065

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Rho GTPases regulate multiple signaling pathways to control a number of cellular processes during epithelial morphogenesis. To investigate the downstream pathways through which Rho regulates epithelial apical junction formation, we screened a small interfering RNA (siRNA) library targeting 28 known Rho target proteins in 16HBE human bronchial epithelial cells. This led to the identification of the serine-threonine kinase PRK2 (protein kinase C-related kinase 2, also called PKN2). Depletion of PRK2 does not block the initial formation of primordial junctions at nascent cell-cell contacts but does prevent their maturation into apical junctions. PRK2 is recruited to primordial junctions, and this localization depends on its C2-like domain. Rho binding is essential for PRK2 function and also facilitates PRK2 recruitment to junctions. Kinase-dead PRK2 acts as a dominant-negative mutant and prevents apical junction formation. We conclude that PRK2 is recruited to nascent cell-cell contacts through its C2-like and Rho-binding domains and promotes junctional maturation through a kinase-dependent pathway.

Apical junctions, including tight and adherens junctions, are important for epithelial cell-cell adhesion, selective permeability, and apical-basal polarity. The formation of apical junctions is therefore essential for epithelia to regulate tissue integrity and homeostasis. Tight junctions and adherens junctions form at the apical margin of the lateral membrane in vertebrate epithelial cells through the interactions of transmembrane junctional proteins. Tight junctions principally consist of the transmembrane proteins occludin and the claudin family, while adherens junctions are principally composed of E-cadherin (2, 29). Additional transmembrane proteins, including nectins, JAM (junctional adhesion molecule), and tricellulin, also contribute to apical junctions. Junctional transmembrane proteins associate via their cytoplasmic domains with a large number of adaptor and signaling proteins and with the actin cytoskeleton (21, 23).

Epithelial apical junction formation is initiated by the *trans* interaction of E-cadherin molecules, which results in the stabilization of E-cadherin puncta at nascent cell-cell contacts, referred to as spot-like or primordial junctions (1). Primordial junctions contain many of the proteins found in mature adherens junctions, including the catenins, as well as the tight junction protein ZO-1 (4, 39). The formation of primordial junctions depends on actin polymerization, and E-cadherin puncta are stabilized at cell-cell contacts by interacting with actin filaments (10, 17). The formation of mature apical junctions, consisting of distinct tight and adherens junctions, requires the recruitment of additional tight junction proteins and the reorganization of the actin cytoskeleton to form the characteristic perijunctional actin belt, a process that requires actomyosin contractility (17, 39, 47). Epithelial apical junctions can thus be regulated by a number of cellular processes, including the expression and trafficking of junctional proteins and the organization of the actin cytoskeleton (13, 44). Many signaling pathways have been implicated in the regulation of epithelial apical junctions, including those controlled by the Rho GTPase family members Rho, Rac, and Cdc42 (15, 34).

Rho plays a particularly important role in epithelial morphogenesis, as one of its target proteins, Rho kinase (ROCK), is a key regulator of myosin II-dependent actomyosin contractility (32). ROCK activates myosin II by inhibiting MLC (myosin light chain) phosphatase, leading to increased MLC phosphorylation. During embryogenesis, apical constriction of epithelial cells, as a result of apically localized myosin II activity, contributes to cell invagination events. In the *Drosophila melanogaster* embryo, for example, localized activation of Rho has been shown to control apical constriction during gastrulation and spiracle cell invagination (19, 37). Another key morphogenetic event during embryogenesis is the sealing of epithelial sheets, and Rho, acting through myosin II, is required for the elongation of leading-edge cells during *Drosophila* dorsal closure (12, 16).

Evidence that Rho regulates apical junction formation in mammalian epithelial cells has come from experimental manipulation of Rho activity using bacterial C3 transferase or the expression of mutant Rho proteins in numerous cell types, including MDCK kidney epithelial cells, keratinocytes, Eph4 mammary epithelial cells, T84 intestinal cells, MCF7 breast carcinoma cells, and HCT116 colon carcinoma cells (6, 26, 33, 38, 40, 46). Investigation of the downstream signaling pathways through which Rho regulates apical junctions has principally focused on ROCK. The inhibition of ROCK in T84 cells prevents apical junction formation, and in MCF7 breast carcinoma cells, it results in reduced E-cadherin accumulation at cell-cell contacts (36, 43). ROCK is believed to promote reorganization of the characteristic perijunctional apical actin belt, which supports apical junction formation/stabilization in po-

* Corresponding author. Mailing address: Cell Biology Program, Memorial Sloan-Kettering Cancer Center, 1274 York Ave., New York, NY 10065. Phone: (212) 639-2387. Fax: (212) 717-3604. E-mail: halla@mskcc.org.

‡ Present address: Angiogenesis Laboratory, CIPM, Portuguese Institute of Oncology, Lisbon, Portugal.

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larized epithelial cells, through actomyosin contractility (17, 38, 47). However, ROCK inhibition has no effect on adherens junction formation in MDCK or HCT116 cells, suggesting that alternative and/or redundant pathways downstream of Rho are active in different cell types (33).

In addition to ROCK, more than 20 other Rho target proteins have been described. In the present study, we report a systematic analysis of Rho signaling pathways regulating apical junction formation in 16HBE human bronchial epithelial cells, an immortalized but nontransformed cell line derived from the epithelium of the lung airway (9). Understanding the pathways that regulate the integrity of the lung epithelium is of great importance, as loss of epithelial integrity is a characteristic feature of lung diseases, including cancer and chronic obstructive pulmonary disease (45). In this study, we identify the Rho target PRK2 (protein kinase C-related kinase 2) as a regulator of apical junction formation in human bronchial epithelial cells.

MATERIALS AND METHODS

Reagents and antibodies. Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The primary antibodies used were RhoA (clone 26C4) and RhoA/C (rabbit polyclonal, sc-179) from Santa Cruz Biotechnology (Santa Cruz, CA); occludin (rabbit polyclonal), ZO-1 (clone 1A12), ZO-1 (rabbit polyclonal), and E-cadherin (clone ECCD-2) from Invitrogen (Carlsbad, CA); E-cadherin (clone 34) and PRK2 (clone 22) from BD Transduction (Lexington, KY); phospho-PRK1 (Thr774)/PRK2 (Thr816) (rabbit polyclonal) from Cell Signaling (Beverly, MA); α-tubulin (clone YL1/2) from AbD Setotec (Raleigh, NC); β-actin (clone AC-74) and FLAG (clone M2) from Sigma-Aldrich; hemagglutinin (HA; clone 3F10) from Roche; and myc (clone 9E10) from Cancer Research UK (London, United Kingdom). Alexa Fluor 488- and 568-conjugated secondary antibodies and Alexa Fluor 488-conjugated phalloidin were from Invitrogen. Aminomethylcoumarin acetate (AMCA)-, fluorescein isothiocyanate (FITC)-, and Cy3-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Cell culture and transfection. 16HBE14o− cells were provided by Dieter Gruenert (California Pacific Medical Center, San Francisco, CA) and were cultured in minimal essential medium (MEM) plus GlutaMAX (Invitrogen) supplemented with 10% BenchMark FBS (Gemini Bio-Products, West Sacramento, CA) and penicillin (100 U/ml)–streptomycin (100 μg/ml) (Invitrogen) at 37°C in 5% CO_2. Transfections were carried out by seeding cells at low density (1.5×10^4 cells/cm^2, 10 to 20% confluence) and allowing them to adhere overnight. Small interfering RNA (siRNA; 50 nM) was transfected in medium without antibiotics, using 100 pmol siRNA and 5 μl Lipofectamine LTX (Invitrogen) per 1.2×10^5 cells. For DNA transfection, 5 μl Lipofectamine LTX and 200 ng plasmid DNA were used per 1.2×10^5 cells. For retroviral infection, 16HBE cells were seeded as described above and then incubated overnight in growth medium containing retroviral particles produced in HEK293T cells and supplemented with 8 μg/ml Polybrene (hexadimethrine bromide). Two days after infection, stable pools were selected using 1.5 μg/ml puromycin (Invitrogen). For calcium switch experiments, cells were washed extensively in PBS without calcium, incubated in low-calcium medium for 4 h, and then switched to normal growth medium containing calcium. Low-calcium medium was prepared using Dulbecco's modified Eagle's medium (DMEM) without calcium chloride (Invitrogen) and supplemented with 10% FBS pretreated with Chelex 100 resin (Bio-Rad, Hercules, CA).

HEK293T cells (ATCC, Manassas, VA) were cultured in DME-HG (high-glucose DMEM) plus sodium pyruvate supplemented with 10% FBS and penicillin (100 U/ml)–streptomycin (100 μg/ml) (Invitrogen) at 37°C in 5% CO_2. For transfection, cells were seeded at 3×10^4 cells/cm^2 and allowed to adhere overnight. One microgram plasmid DNA per 3×10^5 cells was transfected using 5 μl Lipofectamine 2000 (Invitrogen). For retroviral particle production, cells were triply transfected with vesicular stomatitis virus G (VSV-G), Gag-Pol, and the pBABE vector of interest, and at 6 h posttransfection, the medium was changed to 16HBE growth medium for 24 h to collect viral particles.

siRNA reagents. siRNAs were from Thermo Fisher Scientific (Lafayette, CO) and included RhoA SMARTpool M-003860-03, RhoA duplex1 D-003860-01, RhoA duplex2 D-003860-02, RhoA duplex3 D-003860-03, RhoA duplex4 D-003860-04, RhoC SMARTpool M-008555-01, PRK2 duplex1 D-004612-03, PRK2 duplex2 D-004612-10, and siControl (custom sequence, GGAAAUUAU ACAAGACCAA). Additional SMARTpool reagents used for screening are listed in Table 1.

TABLE 1. SMARTpool siRNAs (Thermo Fisher Scientific) that target the indicated genes (with NCBI GeneID) and were used for screening

Gene name	GeneID	Alternative name(s)	SMARTpool catalog no.
CDKN1B	1027	p27, Kip1	M-003472-00
CIT	11113	Citron	M-004613-00
CNKSR1	10256	CNK1	M-012217-01
CNKSR2	22866	CNK2	M-020433-00
CNKSR3	154043	CNK3	M-018546-02
DAAM1	23002		M-012925-00
DGKG	1608	DGKγ	M-006715-01
DGKQ	1609	DGKθ	M-005079-02
DIAPH1	1729	DRF1	M-010347-02
DIAPH2	1730	DRF2	M-012029-01
DIAPH3	81624	DRF3	M-018997-01
FLNA	2316	Filamin A	M-012579-01
KTN1	3895	Kinectin 1	M-010605-01
MAP3K1	4214	MEKK1	M-003575-02
MPRIP	23164	M-RIP	M-014102-01
PITPNM1	9600		M-019888-00
PKN1	5585	PRK1	M-004175-02
PKN2	5586	PRK2	M-004612-03
PKN3	29941		M-004647-01
PLCG1	5335	PLCγ1	M-003559-01
PLD1	5537		M-009413-00
PLXNB1	5364	Plexin B1	M-019590-01
PPP1R12A	4659	MBS	M-011340-01
RHPN1	114822	Rhophilin 1	M-015163-01
RHPN2	85415	Rhophilin 2	M-016798-00
ROCK1	6093		M-003536-02
ROCK2	9475		M-004610-02
RTKN	6242	Rhotekin	M-015055-01

DNA constructs. Mouse RhoA and RhoC cDNAs were obtained from ATCC and subcloned into the pRK5myc expression vector. Mouse PRK2 (mPRK2) was obtained from RZPD (Deutsches Resourcenzentrum für Genomforschung, Germany) and subcloned into the pBABE-HA and pRK5myc expression vectors. Note that the clone used (clone IRAV p968C10112D6) contains a deletion of 11 amino acids (Gln 32 to Gln 42) compared to NCBI reference sequence NM_178654.4. mPRK2(K685M) and mPRK2(D781A) were made by PCR amplification using primers containing the appropriate point mutations. mPRK2(A66K,A155K) was made by carrying out 2 rounds of PCR amplification with primers containing the appropriate point mutations. mPRK2ΔC2 contains a deletion of amino acids 381 to 462 and was made by overlap extension PCR using appropriate primers to amplify residues 1 to 381 and 463 to 983. All primers were purchased from Sigma-Genosys. All constructs were sequence verified.

Immunoprecipitation and Western blotting. 16HBE cell lysates were prepared by scraping cells in protein sample buffer (2% SDS, 100 mM dithiothreitol, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.1% bromophenol blue) and boiling for 5 min at 100°C. For immunoprecipitation, transfected HEK293T cells were lysed in immunoprecipitation buffer (1% NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 2 mM phenylmethylsulfonyl fluoride and Complete protease inhibitor tablet (Roche), and cell debris was pelleted by centrifugation at 13,000 rpm and 4°C for 10 min. The soluble fraction was incubated at 4°C with primary antibody for 1 h, followed by incubation with protein G Sepharose beads (Sigma-Aldrich) for 1 h. The beads were washed extensively with immunoprecipitation buffer and boiled in sample buffer. Proteins were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and incubated with the appropriate primary antibodies. Proteins were visualized using horseradish peroxidase-conjugated secondary antibodies (Dako, Carpinteria, CA) and enhanced chemiluminescence (ECL) detection reagents (GE Healthcare, Waukesha, WI).

Microscopy. 16HBE cells grown on glass coverslips were fixed in 3.7% (vol/vol) formaldehyde for 15 min and permeabilized in 0.5% (vol/vol) Triton X-100 for

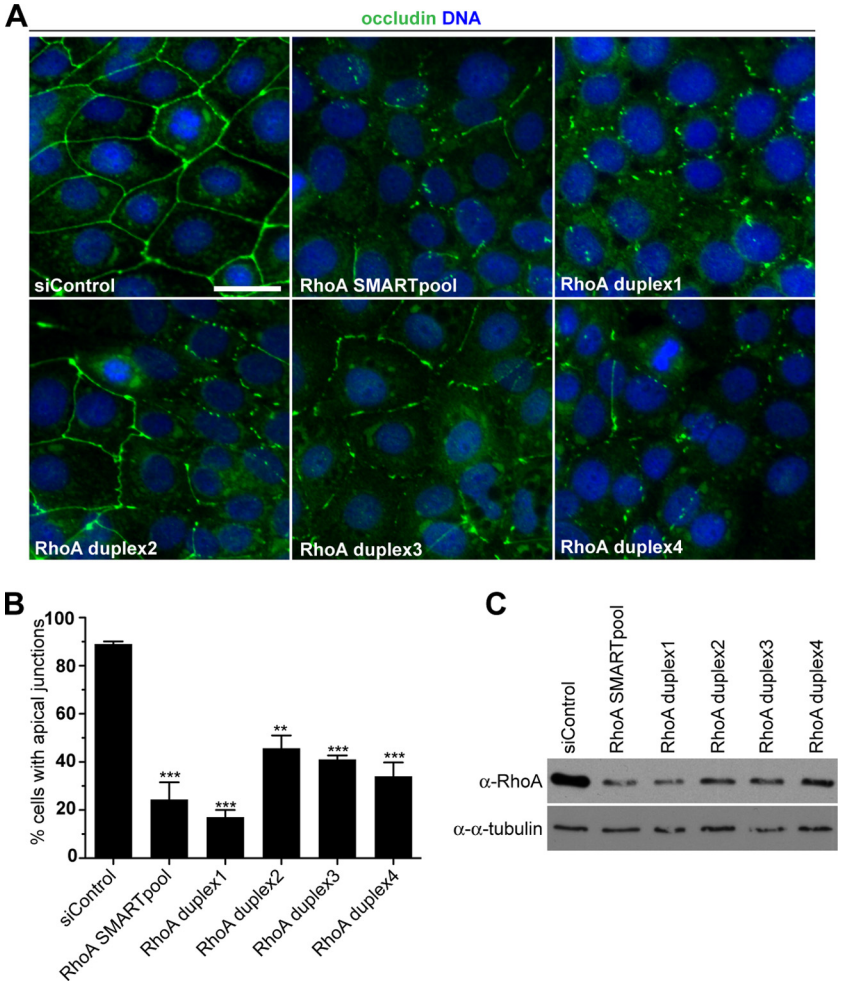

FIG. 1. RhoA regulates apical junction formation in bronchial epithelial cells. 16HBE cells were seeded at low density and transfected with the indicated siRNAs. (A) Cells were fixed at 3 days posttransfection and stained with antioccludin (green) to visualize tight junctions and Hoechst stain (blue) to visualize nuclei. Scale bar shows 20 μm for all images. (B) Quantification of apical junction formation from 3 independent experiments (see Materials and Methods). Error bars, SEM; ***, $P < 0.001$; **, $P < 0.01$. (C) At 3 days posttransfection, cell lysates were prepared and analyzed by Western blot assay with the indicated antibodies.

5 min. Primary and secondary antibody incubations were carried out for 1 h at room temperature. Coverslips were mounted with fluorescent mounting medium (Dako) and visualized using a Zeiss AxioImager.A1 fluorescence microscope with 40× 0.75 numerical aperture (NA) and 63× 1.4 NA objectives (Zeiss, Thornwood, NY), using a Hammamatsu ORCA-ER 1394 C4742-80 digital camera (Bridgewater, NJ) and AxioVision software (Zeiss).

Apical junction quantification. For each sample, 12 random nonoverlapping images were taken at ×40 magnification (~400 cells) and apical junction formation was quantified using the manual count function of Metamorph image analysis software (Universal Imaging, West Chester, PA). Cells with a continuous ring of occludin or ZO-1 at cell-cell contacts were scored as having intact apical junctions. Cells with punctate or discontinuous occludin or ZO-1 at cell-cell contacts were scored as not having apical junctions. The results were analyzed using Prism (GraphPad Software, San Diego, CA). Standard errors of the means (SEM) are shown with error bars, and significance values have been calculated using a two-tailed unpaired *t* test at the 95% confidence interval.

RESULTS

RhoA regulates apical junction formation in bronchial epithelial cells. To determine whether Rho is required for apical junction formation in bronchial epithelial cells, RhoA expression was downregulated in 16HBE cells by RNA interference (RNAi). Cells were seeded at low density and transfected with a SMARTpool siRNA mixture consisting of 4 distinct siRNA duplexes targeting RhoA or with a control siRNA (siControl). Apical junction formation was assessed at 3 days posttransfection by staining with antibodies against the tight junction proteins occludin and ZO-1. The majority of control cells formed apical junctions, defined as a continuous ring of occludin and ZO-1 at cell-cell contacts. However, a significant number of RhoA-depleted cells did not form apical junctions and showed only weak punctate staining of occludin and ZO-1 at cell-cell contacts (Fig. 1A and B, and data not shown). To assess the specificity of this phenotype, 16HBE cells were transfected with the 4 individual siRNA duplexes comprising the RhoA SMARTpool. All 4 siRNA duplexes downregulated RhoA expression and resulted in defective apical junction formation (Fig. 1A to C), showing that this phenotype is a specific consequence of loss of RhoA expression. The RhoA siRNAs used do not affect the expression of RhoC, Rac1, or Cdc42 (data not shown).

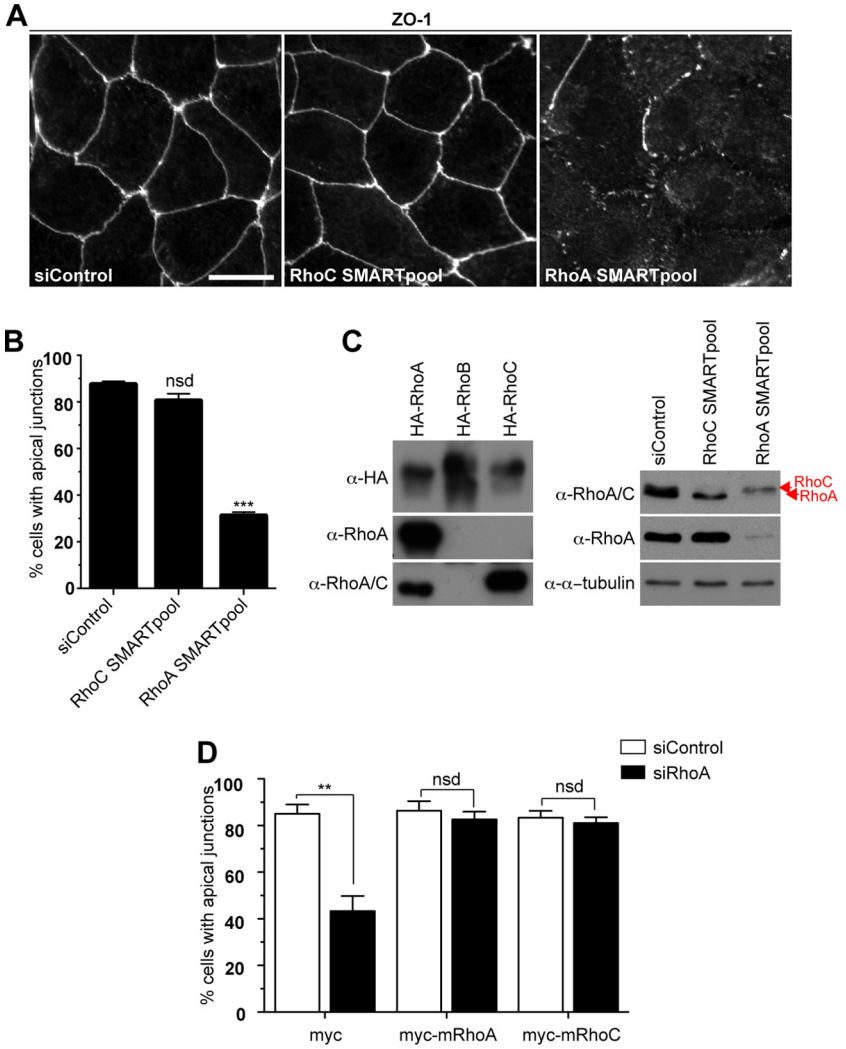

FIG. 2. RhoC acts redundantly with RhoA to regulate apical junction formation. (A) 16HBE cells were seeded at low density and transfected with the indicated siRNAs. At 3 days posttransfection, cells were fixed and stained with anti-ZO-1 antibody. Scale bar shows 20 μm for all images. (B) Quantification of tight junction formation from 3 independent experiments (see Materials and Methods). Error bars, SEM; nsd, no significant difference; ***, $P < 0.001$. (C) RhoA expression is approximately 4-fold higher than RhoC expression in 16HBE cells. Left: lysates from HEK293T cells transfected with HA-tagged Rho GTPases were analyzed by Western blot assay with the indicated antibodies. Note that the anti-RhoA/C antibody binds with greater affinity (approximately 2-fold) to RhoC than to RhoA and does not recognize RhoB. Right: lysates from 16HBE cells transfected with the indicated siRNAs were analyzed by Western blot assay with the indicated antibodies. Note that endogenous RhoA (second lane, lower band) is recognized more strongly (approximately 2-fold) than RhoC (third lane, upper band) with the anti-RhoA/C antibody. (D) Expression of mouse RhoA or mouse RhoC rescues apical junction formation after depletion of endogenous RhoA. 16HBE cells seeded at low density were transfected with myc-tagged RhoA, myc-tagged RhoC, or a control plasmid. Six hours later, cells were transfected with RhoA siRNA duplex2 or siControl. Apical junction formation was analyzed at 3 days posttransfection. At least 100 myc-positive cells per condition from 3 independent experiments were analyzed. Error bars, SEM; nsd, no significant difference; **, $P < 0.01$.

The closely related Rho family member RhoC is 91% identical to RhoA; however, there have been reports of functional differences between RhoA and RhoC. For example, overexpression of RhoC but not of RhoA resulted in disruption of adherens junctions in colon carcinoma cells as a result of ROCK-dependent actomyosin contraction (33). RNAi-mediated depletion of RhoC had no significant effect on apical junction formation in 16HBE cells (Fig. 2A and B), raising the possibility that RhoA and RhoC have nonredundant functions in these cells. Western blot analysis with an antibody that recognizes RhoA and RhoC but with an approximately 2-fold-higher affinity for RhoC (Fig. 2C, left) revealed that RhoA expression is around 4-fold higher than RhoC expression in 16HBE cells (Fig. 2C, right). Furthermore, exogenous expression of either mouse RhoA or mouse RhoC, which are resistant to depletion by RhoA siRNA duplex2, was able to rescue the apical junction defect caused by depletion of RhoA (Fig. 2D). Staining with an antimyc antibody to identify transfected cells showed that RhoA and RhoC were expressed at similar levels in these experiments (data not shown). We conclude that RhoC can act redundantly with RhoA to regulate apical junction formation but, due to its greater abundance, RhoA is the predominant isoform regulating apical junction formation in 16HBE bronchial epithelial cells.

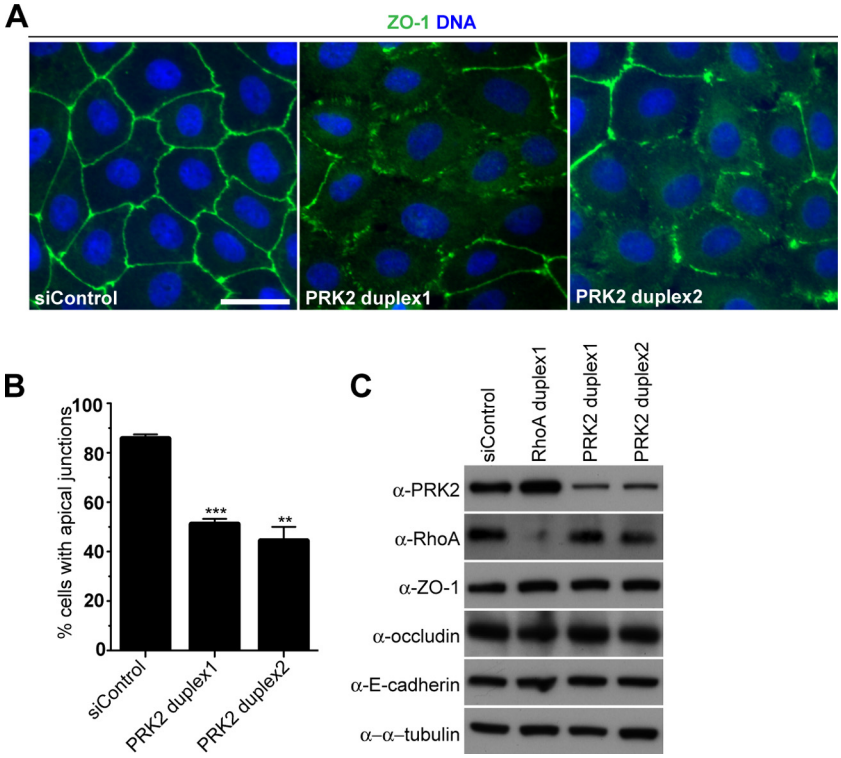

FIG. 3. The Rho target PRK2 regulates apical junction formation. 16HBE cells were seeded at low density and transfected with the indicated siRNAs. (A) At 3 days posttransfection, cells were fixed and stained with anti-ZO-1 (green) and Hoechst stain (blue). Scale bar shows 20 μm for all images. (B) Quantification of apical junction formation from 3 independent experiments (see Materials and Methods). Error bars, SEM; ***, $P < 0.001$; **, $P < 0.01$. (C) At 3 days posttransfection, cell lysates were prepared and analyzed by Western blot assay with the indicated antibodies.

The Rho target protein PRK2 regulates apical junction formation in bronchial epithelial cells. Rho GTPases interact with and regulate specific target proteins. To identify target proteins acting downstream of RhoA in 16HBE cells, a SMARTpool siRNA library targeting 28 known Rho targets was screened (Table 1). One Rho target protein, PRK2, was found to be required for apical junction formation (Fig. 3). Depletion of PRK2 caused a phenotype similar to that of depletion of RhoA, resulting in weak punctate staining of ZO-1 and occludin at cell-cell contacts but no change in the expression level of junctional proteins (Fig. 3 and data not shown). Two of the siRNA duplexes comprising the PRK2 SMARTpool were efficient at downregulating PRK2 expression, and both resulted in defective apical junction formation, suggesting that the effect is specific (Fig. 3). A SMARTpool targeting the closely related PRK1 had no significant effect on junctions. However, when a phospho-PRK antibody that recognizes an identical epitope in the two isoforms is used, PRK1 expression levels are around 2- to 3-fold lower than PRK2 levels in these cells (Fig. 4C), and so, it is possible they share similar activities.

PRK2 is a direct target of RhoA during apical junction formation. To determine whether PRK2 is acting as a direct target of RhoA during apical junction formation, rescue experiments were carried out with a Rho binding-defective mutant. An alanine-to-lysine mutation was introduced into both the HR1a and HR1b GTPase-binding domains of mouse PRK2 to generate mPRK2(A66K,A155K). Based on structural studies of the related protein PRK1, these point mutations are predicted to prevent GTPase binding (27). The results of co-immunoprecipitation experiments confirmed that wild-type mPRK2 interacts with a constitutively activated version of RhoA (L63RhoA) but mPRK2(A66K,A155K) does not (Fig. 4B). There are reports that PRK2 interacts with Rac, in addition to Rho; however, we detected only a weak interaction between mPRK2 and constitutively activated Rac1 (L61Rac1) (Fig. 4B). Human PRK2 shows the same relative binding affinities to RhoA and Rac1 as mouse PRK2 (data not shown). Human PRK2 also interacts similarly with L63RhoC and L63RhoA (data not shown), consistent with RhoC being able to rescue depletion of RhoA (Fig. 2). 16HBE cells were infected with pBABE retroviral vectors containing HA-tagged mPRK2, mPRK2(A66K,A155K), or empty vector control, and stable pools selected with puromycin. Cells were seeded at low density and transfected with PRK2 siRNA duplex1, which contains 4 mismatches with the mouse PRK2 sequence, or with siControl. The expression of wild-type mPRK2 rescued apical junction formation, but the expression of the RhoA-binding-mutant mPRK2(A66K,A155K) did not (Fig. 4D and E). Although the expression level of mPRK2(A66K,A155K) was lower than the expression level of wild-type mPRK2 in the stably expressing cells used (Fig. 4C, HA blot), mPRK2 (A66K,A155K) was nevertheless expressed at higher levels than endogenous PRK2, as determined by blotting with a phospho-PRK antibody that recognizes both endogenous human and exogenous mouse PRK2 (Fig. 4C, phospho-PRK blot). We

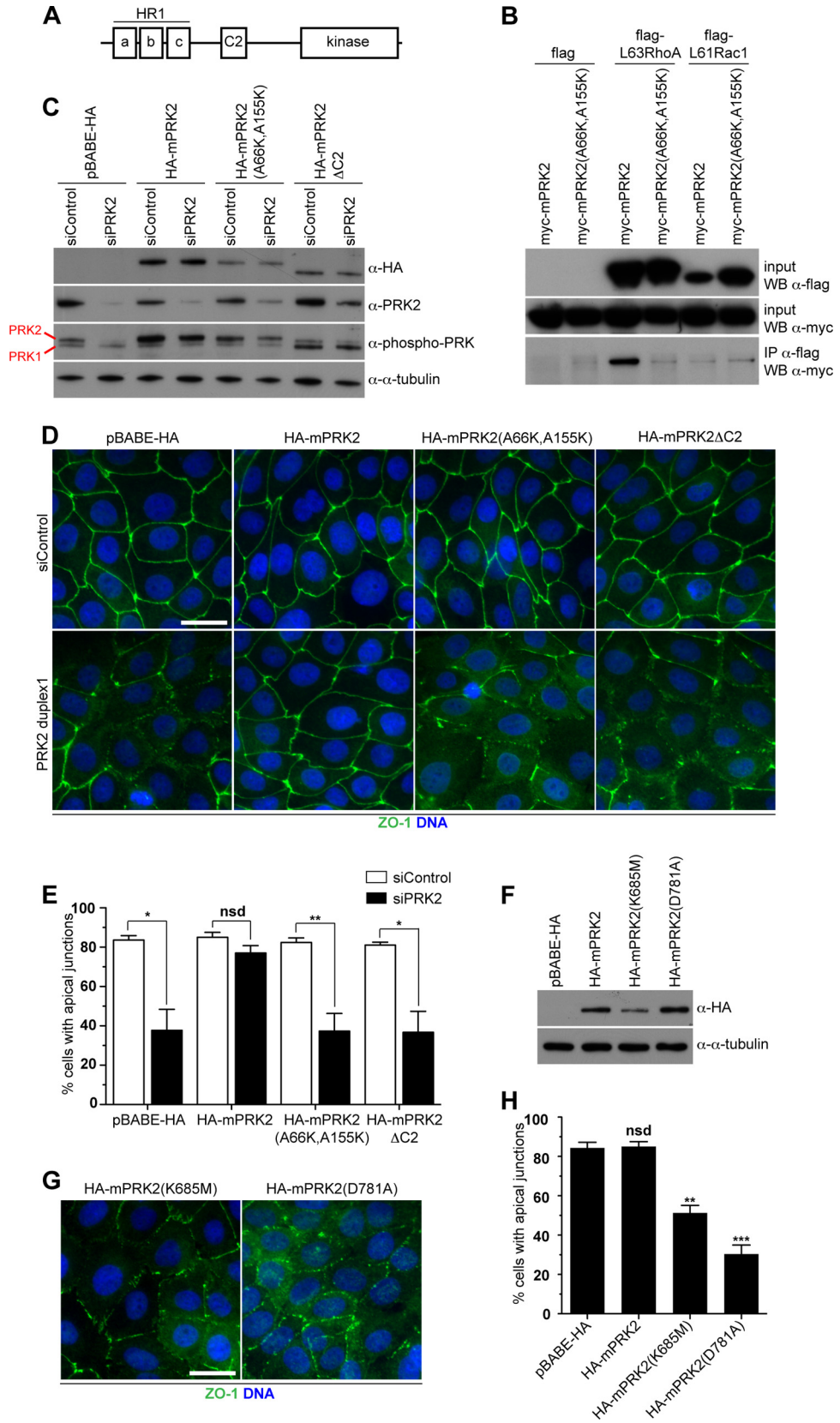

FIG. 4. PRK2 function is RhoA-dependent, kinase-dependent, and requires its C2-like domain. (A) Domain organization of PRK2, including HR1 (homology region 1) GTPase-binding domains, a C2-like domain, and a serine-threonine kinase domain. (B) Wild-type mouse PRK2 (mPRK2) but not mPRK2(A66K,A155K) interacts with L63RhoA in coimmunoprecipitation experiments when overexpressed in HEK293T cells.

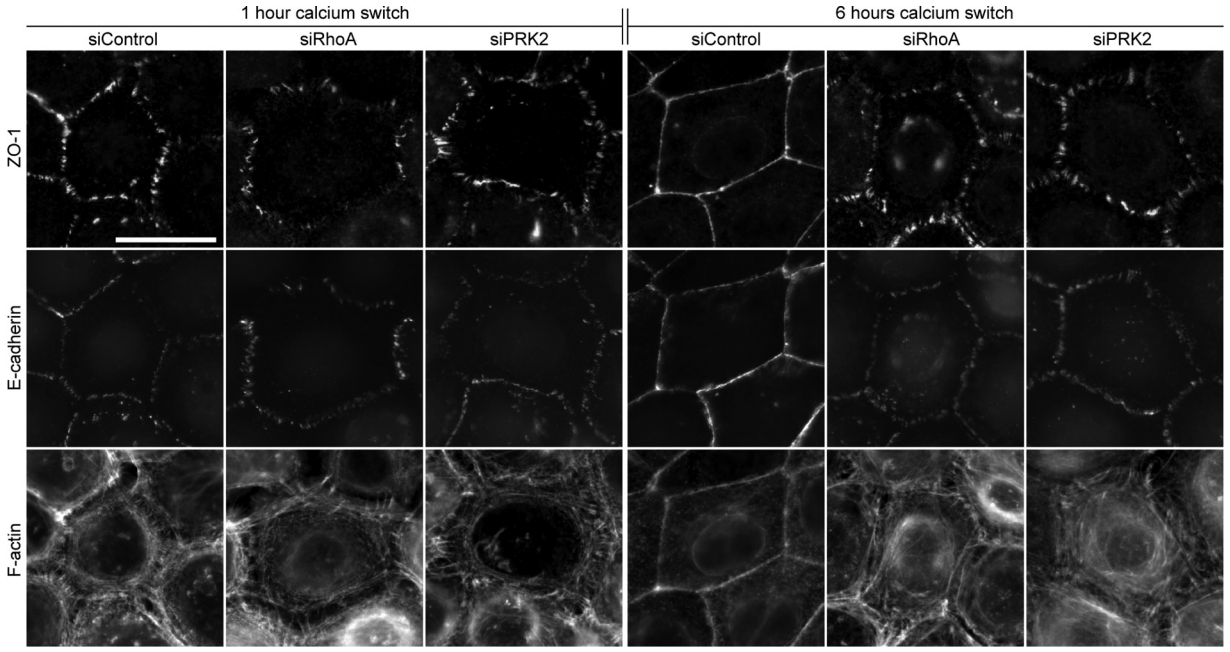

FIG. 5. The RhoA-PRK2 signaling pathway regulates the maturation of primordial junctions into apical junctions. Confluent monolayers of 16HBE cells transfected with RhoA siRNA duplex1, PRK2 siRNA duplex1, or siControl were subjected to calcium switch-induced junction formation. Cells were fixed at 1 h (left) or 6 h (right) after calcium switch and stained with anti-ZO-1, anti-E-cadherin, and Alexa Fluor 488-phalloidin to visualize actin filaments. Scale bar shows 20 μm for all images.

conclude that PRK2 is a direct RhoA target required for apical junction formation in 16HBE cells.

PRK2 function is kinase dependent and requires its C2 domain. In addition to its Rho-binding domain, PRK2 contains a C2-like domain and a kinase domain (Fig. 4A). To determine which domains are required for PRK2 function, additional mutants were generated. mPRK2ΔC2 contains a deletion of amino acids 381 to 462, corresponding to the C2-like domain. When stably expressed in 16HBE cells, mPRK2ΔC2 was unable to rescue apical junction formation after knockdown of endogenous PRK2 (Fig. 4C to E), showing that the C2-like domain is required for PRK2 function during apical junction formation.

Two kinase-dead mutants of mPRK2 were generated. mPRK2 (K685M) contains a mutation in the conserved lysine residue required for ATP binding, and this residue has been shown to be important for PRK2 kinase activity (41). mPRK2(D781A) contains a mutation in the conserved aspartate residue required for phosphate transfer, and mutation of this residue has been shown to be important for kinase activity in AGC family kinases (8). Both kinase-dead mutants acted as dominant negatives in 16HBE cells and inhibited apical junction formation (Fig. 4F to H), indicating that PRK2 function is kinase dependent.

RhoA and PRK2 regulate the maturation of primordial junctions into apical junctions. The apical junctional complex of epithelial cells consists of tight junctions, adherens junctions, and the associated perijunctional actin ring. We have previously described the mechanism of apical junction formation in 16HBE cells by using a calcium switch to induce junction formation (42). Confluent monolayers of 16HBE cells were incubated in low-calcium medium to disassemble junctions, followed by the addition of calcium to stimulate junction formation. Upon the addition of calcium, 16HBE cells first formed primordial junctions, consisting of punctate accumulation of E-cadherin complexes, including ZO-1, at nascent cell-cell contacts (Fig. 5, 1 h). Junctional maturation then resulted in the formation of apical tight and adherens junctions, seen as continuous staining of ZO-1 and E-cadherin, respectively, at

WB, Western blotting; IP, immunoprecipitation. (C to E) 16HBE cells were infected with pBABE retroviral vectors expressing HA-tagged mPRK2, HA-tagged mPRK2(A66K,A155K), or HA-tagged mPRK2ΔC2 and selected in puromycin. Stable pools were transfected with PRK2 siRNA duplex1 or siControl and analyzed at 3 days posttransfection. (C) Western blot analysis of cell lysates with the indicated antibodies. Note that the PRK2 antibody does not recognize the mouse PRK2 constructs, whereas the phospho-PRK antibody has been raised against a site that is completely conserved between human and mouse PRK2. The phospho-PRK antibody also recognizes PRK1. Note that mPRK2ΔC2 runs at the same size as endogenous PRK1. (D) Cells were fixed and stained with anti-ZO-1 (green) and Hoechst stain (blue). Scale bar shows 20 μm for all images. (E) Quantification of apical junction formation (see Materials and Methods) from 3 independent experiments. Error bars, SEM; nsd, no significant difference; **, $P < 0.01$; *, $P < 0.02$. (F to H) 16HBE cells stably expressing HA-tagged mPRK2, mPRK2(K685M), mPRK2(D781A), or pBABE-HA empty vector control were analyzed. (F) Western blot analysis of cell lysates with the indicated antibodies. (G) Cells were fixed and stained with anti-ZO-1 (green) and Hoechst stain (blue). (H) Quantification of apical junction formation (see Materials and Methods) from 3 independent experiments. Error bars, SEM; nsd, no significant difference; ***, $P < 0.001$; **, $P < 0.01$.

the cell-cell contact, and this was accompanied by reorganization of cortical actin filaments to form the perijunctional actin ring (Fig. 5, 6 h). 16HBE monolayers depleted of RhoA or PRK2 were able to form primordial junctions, consisting of E-cadherin puncta, but were unable to form mature apical junctions (Fig. 5). We conclude that the RhoA-PRK2 signaling pathway is required for the transition from primordial junctions to mature apical junctions.

PRK2 is recruited to primordial junctions to regulate their maturation into apical junctions. To understand how PRK2 regulates apical junction formation, we determined its localization. PRK2 is recruited to nascent cell-cell contacts and is first seen at primordial junctions 1 h after calcium switch (Fig. 6A, red arrowheads). PRK2 continues to accumulate at cell-cell contacts as primordial junctions mature into apical junctions (Fig. 6A, 2-h and 6-h time points). This recruitment of PRK2 to primordial junctions is consistent with its role in regulating their maturation.

To assess how PRK2 localization at junctions is regulated, we analyzed the localization of the mutants described above. In contrast to wild-type HA-mPRK2, which can be detected at junctions in over 50% of cells, junctional localization of HA-mPRK2ΔC2 could not be detected (Fig. 6B). The localization of the Rho-binding mutant HA-mPRK2(A66K,A155K) at junctions was also significantly reduced (Fig. 6B), although very weak junctional localization was still detected in approximately 20% of cells. To confirm that RhoA binding regulates PRK2 localization, the localization of endogenous PRK2 was determined in RhoA-depleted cells. In contrast to control cells, PRK2 was not detected at primordial junctions in the majority of RhoA-depleted cells at either 1 h or 6 h after calcium switch (Fig. 6C). We conclude that the C2-like domain and the Rho-binding domain of PRK2 are both required for junctional localization.

DISCUSSION

In this study, we sought to investigate the signaling pathways through which Rho regulates apical junction formation in bronchial epithelial cells. A library of SMARTpool siRNAs targeting 28 Rho target proteins was screened in 16HBE cells, and the protein kinase PRK2 identified. PRK2 belongs to a family of 3 serine-threonine kinases, the PKC-related kinase family, also called PKN (protein kinase novel) (24). PRK isoforms show homology to PKC family kinases within their conserved C-terminal kinase domains and contain an N-terminal GTPase-binding domain and a central domain with weak homology to the calcium-dependent phospholipid binding C2 domain of PKC (Fig. 4A). The GTPase-binding domain, also called the HR1 (homology region 1), contains 3 tandem repeats of approximately 70 amino acids encoding antiparallel coiled-coil domains (referred to as HR1a to -c), which form independent GTPase-binding modules. In the case of PRK1 at least, only HR1a and HR1b bind to Rho GTPases (11). Initial studies of PRK1 found it to interact with active RhoA, RhoB, and RhoC but not Rac1, and when PRK2 was cloned, a similar specificity for Rho but not Rac was observed (3, 31). PRK2 has since been shown to interact with Rac, as well as Rho (41); however, we found only a very weak interaction between PRK2 and a constitutively active mutant of Rac1 in coimmunoprecipitation experiments. Point mutations were introduced into the HR1a and HR1b domains to prevent binding of PRK2 to active RhoA, and this mutant failed to rescue apical junction formation when endogenous PRK2 was depleted, showing that PRK2 acts as a RhoA target during apical junction formation.

A single PRK/PKN homolog exists in *Drosophila*, and there is evidence that it regulates epithelial morphogenesis. Null mutants of *Drosophila* PKN show defects in dorsal closure, a developmental process in which leading-edge cells of the epidermis elongate along the dorsal-ventral axis until they meet at the dorsal midline (20). Dorsal closure requires dynamic regulation of cell-cell adhesion and actomyosin-dependent cell shape changes, and PKN has been proposed to regulate this downstream of Rho (5). Biological roles for mammalian PRK proteins have not been clearly elucidated; however, overexpression studies have provided some clues to PRK function. RhoB recruits PRK1 to endosomes and has been suggested to regulate trafficking in HeLa cells (14). PRK2 has been implicated in the regulation of cadherin-dependent cell-cell adhesion in keratinocytes, as overexpression of PRK2 resulted in increased localization of E-cadherin at cell-cell contacts and increased adhesiveness (7). In this study, we demonstrate that PRK2 is required for the transition from primordial junctions to mature apical junctions. 16HBE cells depleted of PRK2 were able to form primordial junctions, consisting of punctate E-cadherin complexes at nascent cell-cell contacts, but these primordial junctions did not mature into apical junctions, consisting of tight and adherens junctions and the associated perijunctional actin filaments. We previously showed that PRK2 localizes to the midbody during cytokinesis and that PRK2 depletion in HeLa cells results in defective cell division, leading to the formation of multinucleated cells (35). No significant increase in multinucleated cells was observed in 16HBE cells depleted of PRK2, although PRK2 does localize at the midbody of these cells during cytokinesis (not shown).

PRK2 localizes to primordial junctions in 16HBE cells to promote their maturation. Localization of PRK2 at junctions is dependent on its C2-like domain. C2 domains are calcium-dependent phospholipid binding domains first described in classical PKC family members (22). The C2 domain of PRK lacks critical residues for calcium binding and is therefore referred to as a C2-like domain (30). C2-like domains might function as calcium-independent phospholipid binding domains but could also function as protein-protein interaction domains (22). PRK1 interacts with the actin filament binding protein α-actinin, and the interacting region has been mapped to residues 136 to 474, a region that overlaps with the C2-like domain (25). Interestingly, α-actinin localizes to adherens junctions in epithelial cells, raising the possibility that α-actinin recruits PRK2 to junctions (28). However, we found that during apical junction formation, α-actinin associates with cortical actin filaments and that it is only found at the cell-cell contact in mature junctions (not shown), so it is unlikely that α-actinin is responsible for recruiting PRK2 to primordial junctions.

Mutations in the HR1 domains of PRK2 to prevent binding to RhoA significantly reduced the junctional localization of PRK2, showing that RhoA binding contributes to this localization. Fluorescence resonance energy transfer (FRET)-based biosensors have been used to study RhoA activation in epithelial cells as junctions form, and it was found that RhoA

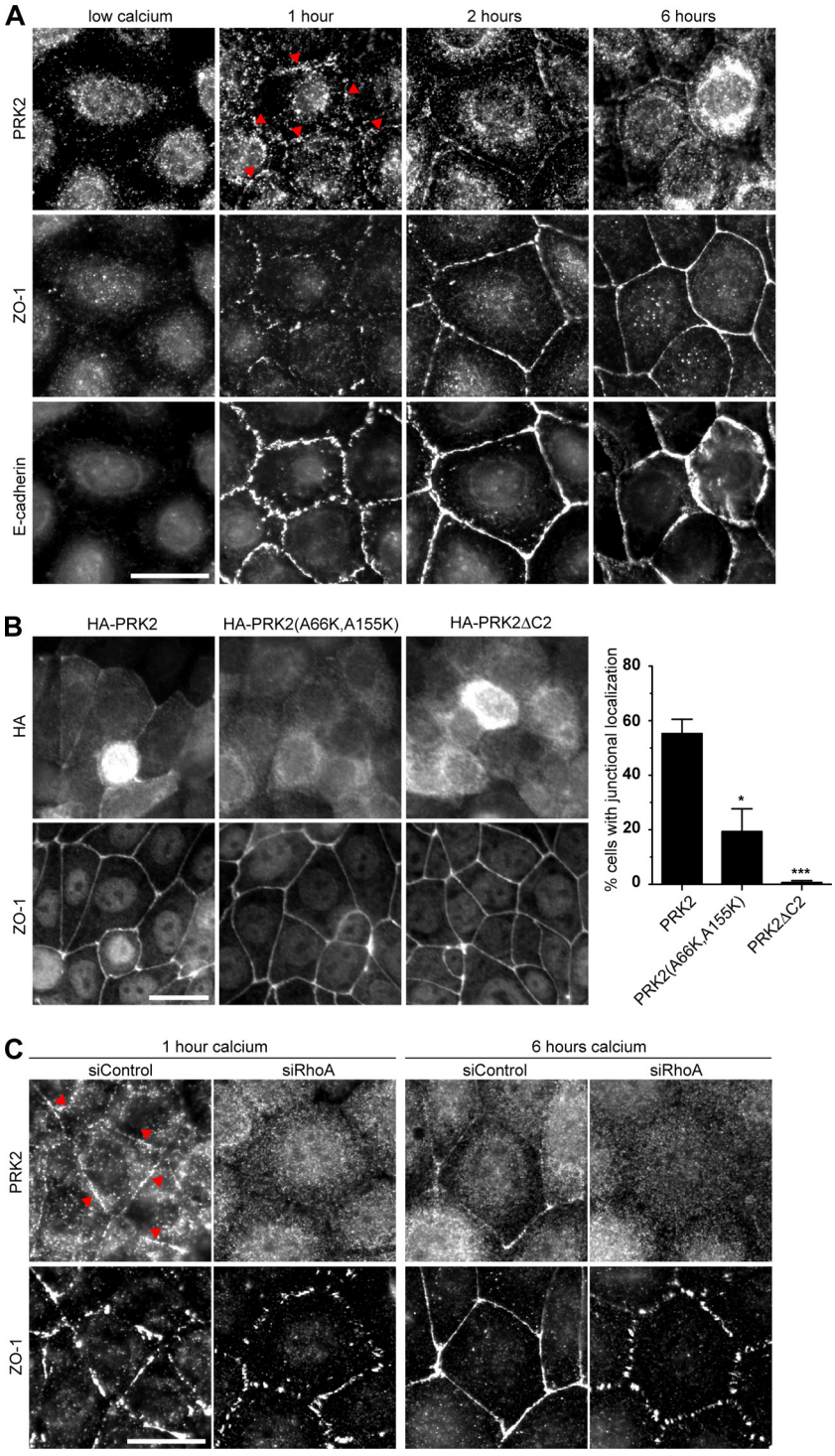

FIG. 6. PRK2 is recruited to primordial junctions to regulate their maturation. (A) 16HBE monolayers were subjected to calcium switch, fixed at the indicated times, and stained with the indicated antibodies. Red arrowheads indicate recruitment of PRK2 to primordial junctions at the 1-h time point. (B) 16HBE cells stably expressing HA-tagged mPRK2 mutants were seeded at low density and analyzed 4 days later by staining with anti-HA and anti-ZO-1 antibodies (images). Junctional localization was quantified (histogram) by analyzing at least 100 cells per experiment from 3 independent experiments and scoring for the presence of HA staining at junctions. Error bars, SEM; ***, $P < 0.001$; *, $P < 0.05$. (C) 16HBE monolayers transfected with siControl or RhoA siRNA duplex1 were subjected to calcium switch-induced junction formation. Cells were fixed at the 1-h and 6-h time points and stained with anti-PRK2 and anti-ZO-1 antibodies. Red arrowheads indicate recruitment of PRK2 to primordial junctions in control cells. Scale bar shows 20 μm for all images.

is activated at nascent cell-cell contacts (46). The Rho binding-defective mutant did, however, show partial junctional localization in approximately 20% of cells, and yet, it completely failed to rescue tight junction formation after knockdown of endogenous PRK2, suggesting that RhoA is also likely to regulate PRK2 function through an additional mechanism. *In vitro* GTP-bound RhoA enhances the kinase activity of PRK2 (41). The binding of RhoA to the N-terminal GTPase-binding domain of PRK1 has been proposed to disrupt an autoinhibitory closed conformation between the N terminus and the kinase domain (18). By analogy, the interaction between active RhoA and PRK2 might allow PRK2 to adopt an open conformation at cell-cell contacts, thus activating the kinase domain and facilitating an interaction between the C2-like domain and as-yet-unidentified proteins/lipids to stably localize PRK2 at maturing junctions.

We recently described two additional serine-threonine kinases required for the formation of mature apical junctions in 16HBE cells, PAK4 and aPKC, both Cdc42 targets (42). Depletion of PRK2, PAK4, or aPKC leads to an apparently identical phenotype in which cells undergo the initial step of junction formation to form primordial junctions but these do not mature into apical junctions. This raises the question of why three separate protein kinase activities would be required for junctional maturation to occur. Apical junction formation requires the coordination of several processes, such as trafficking of proteins to the plasma membrane, regulation of junctional protein complexes, and reorganization of the cortical actin cytoskeleton. It therefore seems likely that PRK2, PAK4, and aPKC regulate different aspects of junction formation by phosphorylating distinct substrates. Future work will be aimed at identifying the relevant substrates of these kinases.

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