Molecular decipherment of Rho effector pathways regulating tight-junction permeability

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We reported recently that the activation of RhoA induced an increase in transepithelial electrical resistance (TER). To clarify effectors of Rho for this RhoA-induced regulation of tight-junction permeability, we introduced two effector-loop mutants of constitutively active RhoA^{V14}, RhoA$^{V14/L40}$ and RhoA$^{V14/C42}$, into Mardin–Darby canine kidney cells in an isopropyl β-D-thiogalactoside-inducible expression system. RhoA^{V14} and the two effector-loop mutants interacted *in vitro* with the Rho-binding domain of Rho-associated kinase, ROKα. Next we examined two parameters of Rho functions, stress-fibre formation and TER elevation, induced by RhoA^{V14}. Stress-fibre formation was induced by RhoA$^{V14/C42}$ but not by RhoA$^{V14/L40}$. On the other hand, TER elevation was induced by neither RhoA$^{V14/L40}$ nor RhoA$^{V14/C42}$. RhoA-associated kinase inhibitor, Y-27632, inhibited both stress-fibre formation and TER elevation induced by RhoA^{V14}. These results demonstrated that RhoA-induced regulation of tight-junction permeability is mediated by Rho-associated kinase and at least one other unidentified effector, the coupling to RhoA being disrupted by mutation at position 40 or 42 in the effector loop.

Key words: MDCK, Rho-associated kinase, small G-protein, stress fibre, TER.

INTRODUCTION

Epithelia form barriers and regulate vectorial transport of ions and solutes. Epithelial cells adhere to each other via three distinct adhesion systems, tight junctions, adherens junctions and desmosomes. Of these, the tight junction is the most apical component and is localized to the interface between apical and basolateral membrane domains [1,2]. It forms not only a boundary in the plasma membrane bilayer that separates the cell surface into biochemically and functionally distinct apical and basolateral membrane domains, but also a barrier to the paracellular diffusion of ions and solutes.

Rho is a member of a subfamily of small GTPases that is thought to be involved in many cellular functions, including the regulation of actin filament reorganization, cell-shape change and gene expression [3,4]. Extracellular stimuli convert inactive GDP-bound Rho into active GTP-bound Rho. Once activated, Rho interacts with its specific effectors, eliciting a variety of biological functions [5]. Recently, several effector proteins that interact only with GTP-bound Rho have been identified, including protein kinase N [6], Rho-associated kinase [7–9], rhophilin [10], rhotekin [11] and mDia1 [12]. Among them, Rho-associated kinase has been reported to be involved with several functions of Rho: the regulation of myosin phosphorylation [13], the formation of stress fibres and focal adhesions [14] and neurite retraction [15,16]. As for tight junction, C3 transferase, which ADP-ribosylates Rho and abrogates the actions of Rho [17], was reported to disrupt tight junctions, suggesting that Rho was involved in tight-junction integrity in epithelial cells [18]. In addition, we recently revealed that induction of constitutively active RhoA increased transepithelial electrical resistance (TER), indicating that Rho plays a role in tight-junction permeability [19]. However, downstream effectors of Rho that regulate tight-junction permeability have not yet been identified. In the present study, we set out to clarify effectors involved in the RhoA-induced regulation of tight-junction permeability by using RhoA effector-loop mutants, and we demonstrated that Rho-associated kinase and at least one other effector were involved in the regulation by RhoA.

MATERIALS AND METHODS

Construction of expression plasmids

The cDNAs of constitutively active RhoA, RhoA^{V14}, and dominant negative RhoA, RhoA^{N19}, were obtained as described previously [15], and the cDNAs of the effector-loop mutants of RhoA^{V14}, RhoA$^{V14/L40}$ and RhoA$^{V14/C42}$, were generated by PCR-mediated mutagenesis [20]. These cDNAs were subcloned into pBluescript SK(+) containing the haemagglutinin (HA) epitope tag sequence at the 5′ end, and inserted into the mammalian expression vector pOPRSVICAT (Stratagene) at the site of *Not*I (pOPRSVI/HA-RhoA$^{V14/L40}$ and HA-RhoA$^{V14/C42}$).

Cell culture and transfection

The Mardin–Darby canine kidney (MDCK) strain II cell line was a generous gift from Dr Keith E. Mostov (University of California, San Francisco, CA, U.S.A.). MDCK cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 4 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin under humidified air containing 5% CO_2 at 37 °C. The MDCK cell line expressing HA-RhoA^{V14} under the control of isopropyl β-D-thiogalactoside (IPTG) has been described previously [19]. To establish the MDCK cell lines of IPTG-inducible HA-RhoA$^{V14/L40}$ and HA-RhoA$^{V14/C42}$, Lac repressor-expressing MDCK cells, established previously [19], were transfected with pOPRSVI/HA-RhoA$^{V14/L40}$ or HA-

Abbreviations used: MDCK, Mardin–Darby canine kidney; TER, transepithelial electrical resistance; HA, haemagglutinin; IPTG, isopropyl β-D-thiogalactoside; RhoA^{V14}, constitutively active RhoA; GST, glutathione S-transferase; RBD, Rho-binding domain; ROKα, RhoA-binding kinase α; SRF, serum-response factor.

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$RhoA^{V14/C42}$ constructs by CellPhect Transfection Kit (Amersham Pharmacia Biotech). Stable transformants were cloned by selection with hygromycin (Wako Corp., Osaka, Japan) and G418 (GIBCO Laboratories Life Technologies).

Protein-interaction assay *in vitro*

Glutathione S-transferase (GST)-associated Rho-binding domain (RBD; amino acids 932–1065) of ROKα (RhoA-binding kinase α; GST-RBD) was prepared as described previously [15]. Purification of recombinant RhoA mutants and their loading with GTP[S] or GDP were as described previously [21]. Following loading with GTP[S] or GDP, 100 ng of RhoA mutant proteins were incubated with gentle agitation for 30 min at 4 °C with 10 μg of GST-RBD, immobilized on glutathione–Sepharose (Amersham Pharmacia Biotech) in 45 mM Tris/HCl (pH 7.5), 4 mM EDTA, 18 mM NaCl, 5 mM $MgCl_2$, 56 μM dithiothreitol and 440 μg/ml BSA. Beads were then washed three times in 50 mM Tris/HCl (pH 7.5), 100 mM NaCl and 5 mM $MgCl_2$. RhoA proteins that remained bound to the beads were eluted by boiling in the loading buffer, subjected to SDS/PAGE (12.5% gel) and detected by immunoblotting with anti-RhoA antibody.

SDS/PAGE and immunoblotting

The cells were stimulated with 5 mM IPTG in the medium for the time indicated. After rinsing the cells briefly with PBS, they were solubilized with RIPA buffer [1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 0.15 M NaCl, 10 mM Hepes (pH 7.4), 25 mM NaF, 1 mM orthovanadate, 2 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.1 μM benzamidine and 1 mM PMSF] at 4 °C for 20 min. Afterwards the cell lysate was centrifuged at 18000 ***g*** for 10 min, and the supernatant was separated by SDS/PAGE (12.5% gel). The proteins were transferred on to a PVDF membrane (Millipore), and HA-Rho proteins were detected with anti-HA antibody using an enhanced chemiluminescence ECL® Western-blotting system (Amersham Pharmacia Biotech).

Fluorescence microscopy

MDCK cells were seeded on to poly-L-lysine-coated glass coverslips in 12-well plates at a density of 1×10^4 cells/well, and cultured for 2 days. For localization of actin filaments, immunofluorescence microscopy was performed as described previously [22]. Briefly, the cells were fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 3% sucrose for 1 h at 4 °C. They were then permeabilized with TPBS/HS (high salt; 10 mM phosphate buffer, pH 7.4, containing 0.1 M NaCl and 0.1% Tween 20), containing 0.1% Triton X-100 at room temperature for 10 min and washed with TPBS/HS twice. They were blocked with 0.5% BSA in TPBS/LS (low salt; PBS containing 0.05% Tween 20) for 1 h at room temperature and rinsed with TPBS/HS. To identify the polymerized actin, they were incubated with 1:100 dilution of Alexa™ 488 phalloidin (Molecular Probes, Eugene, OR, U.S.A.) in TPBS/LS for 1 h at room temperature. They were then washed with TPBS/HS three times and mounted on to a slide glass in PBS containing glycerol and *p*-phenylenediamine dihydrochloride. Cells were photographed at $\times 630$ magnification under a fluorescence confocal microscope.

TER

Y-27632 was a generous gift from Dr T. Akira (Yoshitomi Pharmaceutical Industries, Saitama, Japan). For culture on permeable support, MDCK cells were seeded on cell-culture insert (Falcon 3095) at a density of 3×10^4 cells/filter and cultured for 5 days. After cells had been serum-starved in serum-free medium for 1 h, they were stimulated with or without 5 mM IPTG in the presence or absence of Y-27632. TER was measured with a Millicell-ERS apparatus (Millipore). Calculations for $\Omega \times cm^2$ were made by subtracting values of blank inserts from all samples and multiplying by the area of the monolayer (0.31 cm^2).

RESULTS

Generation of inducible RhoA effector-loop mutants

The effector region of RhoA is crucial for activation of various effector proteins by Rho, and effector-loop mutants of RhoA have been shown to be coupled to distinct effector pathways dependent on the mutation sites [23]. Rho-associated kinase has been shown to be involved in a variety of actions of Rho [4]. We first constructed two effector-loop mutants (E40L and Y42C) of $RhoA^{V14}$ and examined binding *in vitro* of these RhoA mutants to the RBD of Rho-associated kinase, ROKα. As shown in Figure 1, constitutively active RhoA, $RhoA^{V14}$, and its two effector-loop mutants, $RhoA^{V14/L40}$ and $RhoA^{V14/C42}$, interacted with GST-RBD. These three RhoA mutants showed similar affinity for GST-RBD under washing with a stronger ionic strength, although this washing evenly reduced the binding affinities of these mutants for GST-RBD (results not shown). On the other hand, dominant-negative RhoA, $RhoA^{N19}$, failed to interact with GST-RBD, indicating that the interaction of the active RhoA derivatives with RBD is specific.

To reveal effectors for Rho-induced regulation of tight-junction permeability, we established MDCK cell lines, which express HA-$RhoA^{V14/L40}$ and HA-$RhoA^{V14/C42}$ under the control of IPTG. HA-$RhoA^{V14}$, HA-$RhoA^{V14/L40}$ or HA-$RhoA^{V14/C42}$ were expressed by incubation with 5 mM IPTG for up to 6 h in these MDCK cell lines, the expression reaching a maximum level at 9 h (Figure 2). A slight decline in the level of expression of $RhoA^{V14}$ 24 h after IPTG addition was observed. This decline might have been due to proteolytic degradation. When the expression levels of these RhoA mutants were compared with endogenous RhoA, the level of endogenous RhoA was several-fold higher than those of expressed $RhoA^{V14}$, $RhoA^{V14/L40}$ and $RhoA^{V14/C42}$ (results not shown).

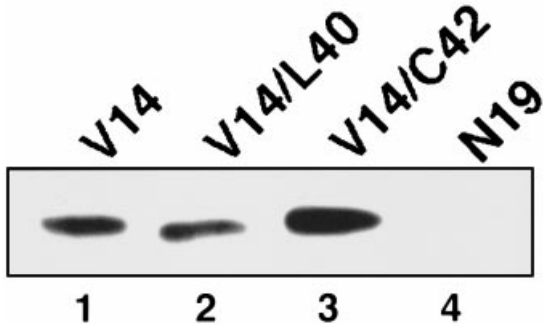

Figure 1 Interaction *in vitro* between RhoA mutants and RBD of ROKα

GTP[S]-loaded $RhoA^{V14}$ (lane 1), $RhoA^{V14/L40}$ (lane 2) and $RhoA^{V14/C42}$ (lane 3), and GDP-loaded $RhoA^{N19}$ (lane 4) were incubated with GST-RBD. Rho proteins bound to GST-RBD were detected with anti-RhoA antibody following elution, SDS/PAGE (12.5% gel) and immunoblotting, as described in the Materials and methods section. The results shown are representative of seven independent experiments that yielded similar results.

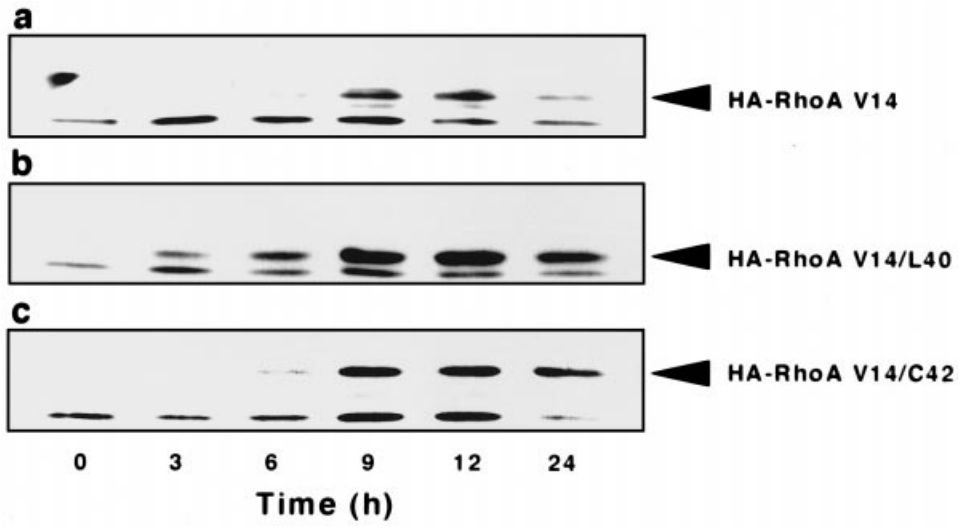

Figure 2 Expression of RhoA mutants induced by IPTG in RhoA mutant-inducible MDCK cells

(**a**) $RhoA^{V14}$-, (**b**) $RhoA^{V14/L40}$- or (**c**) $RhoA^{V14/C42}$-inducible MDCK cells were treated without (0) or with 5 mM IPTG for 3, 6, 9, 12 or 24 h. The cell lysate was separated by SDS/PAGE (12.5% gel) and followed by immunoblotting with anti-HA antibody, as described in the Materials and methods section. The results shown are representative of three independent experiments that yielded similar results.

Regulation of stress-fibre formation and tight-junction permeability by RhoA effector-loop mutants

Stress-fibre formation is one of the prominent functions of Rho [3]. We therefore, examined the effects of RhoA effector-loop mutants on the distribution of F-actin. As shown in Figure 3, IPTG induced stress-fibre formation in $RhoA^{V14}$- and $RhoA^{V14/C42}$-inducible cells, but not in $RhoA^{V14/L40}$-inducible cells, indicating that the RhoA-induced stress-fibre formation was sensitive to mutation at 40 but not at 42.

TER is a parameter of tight-junction ionic permeability in MDCK strain II cells [24]. We demonstrated recently that expression of $RhoA^{V14}$ induced the elevation of TER in MDCK cells [19]. We then examined the ability of effector-loop mutants of RhoA to elevate TER. As shown previously [19], the induction of $RhoA^{V14}$ by IPTG progressively elevated the level of TER up to 24 h, with a lag period of 12 h in $RhoA^{V14}$-inducible cells (Figure 4a). On the other hand, both RhoA effector-loop mutants, $RhoA^{V14/L40}$ and $RhoA^{V14/C42}$, did not affect the TER level up to 24 h (Figures 4b and 4c). Thus the positions of 40 and 42 in the effector loop are crucial for the RhoA-induced regulation of tight-junction permeability.

Involvement of Rho-associated kinase in RhoA-induced regulation of tight-junction permeability

Whereas two effector-loop mutants of $RhoA^{V14}$ were able to interact with Rho-associated kinase, they failed to elevate TER. To reveal the involvement of Rho-associated kinase in RhoA-induced regulation of tight-junction permeability, we examined the effect of a specific Rho-associated kinase inhibitor, Y-27632, on the RhoA-induced elevation of TER. Rho-associated kinase is involved in Rho-mediated stress-fibre formation, and Y-27632 has been shown to inhibit $RhoA^{V14}$-induced stress-fibre formation in HeLa cells [25]. We first examined the effect of Y-27632 on active RhoA-induced stress-fibre formation in MDCK cells. As shown in Figure 5(A), treatment with Y-27632 inhibited the $RhoA^{V14}$- and $RhoA^{V14/C42}$-induced stress-fibre formation, indicating that Y-27632 exerted an effective inhibition of Rho-associated kinase in MDCK cells. Next we examined the effect of Y-27632 on the RhoA-induced regulation of tight-junction permeability. Y-27632 treatment suppressed the $RhoA^{V14}$-induced TER elevation (Figure 5B). Y-27632 did not affect IPTG-induced $RhoA^{V14}$ expression, indicating that the inhibitory effect of Y-27632 on $RhoA^{V14}$-induced TER elevation was not due to suppression of IPTG-induced RhoA expression (results not shown). Therefore, Rho-associated kinase is involved in the RhoA-induced regulation of tight-junction permeability.

DISCUSSION

We demonstrated recently that activation of Rho induced an increase in TER in MDCK cells [19]. We have analysed effectors acting downstream of Rho for the regulation of tight-junction permeability, and showed that Rho-associated kinase and additional effector(s) were involved in the regulation of tight-junction permeability by RhoA.

In this study we used the effector-loop mutants to investigate the role of RhoA. Binding studies demonstrated that mutations at 40 and 42 did not disrupt the interaction between RhoA and Rho-associated kinase, the best-characterized Rho effector kinase. Binding of $RhoA^{V14/L40}$ to Rho-associated kinase was in contrast to that reported by Sahai et al., who showed that this mutant failed to associate with the kinase [23], but the reason for this discrepancy is unclear. Possibly, the assay conditions may be somehow different.

Stress-fibre formation is a prominent action of Rho [3], and Rho-associated kinase has been reported to be involved in RhoA-induced stress-fibre formation [14]. Involvement of Rho-associated kinase in stress-fibre formation was confirmed by our observation that a Rho-associated kinase inhibitor, Y-27632, suppressed the $RhoA^{V14}$-induced stress-fibre formation. We showed here that two effector-loop mutants, $RhoA^{V14/L40}$ and $RhoA^{V14/C42}$, had ability to interact with Rho-associated kinase, but the former mutant could not induce stress-fibre formation,

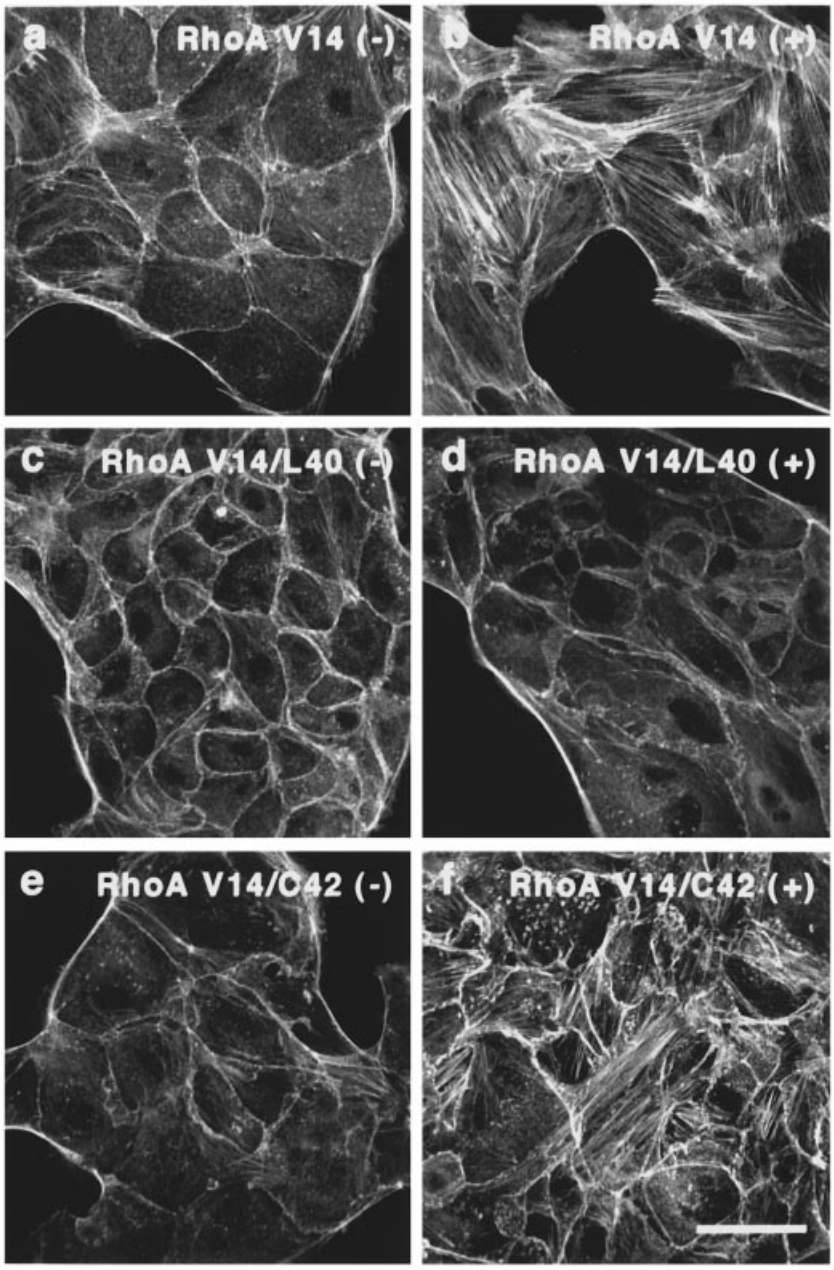

Figure 3 F-actin distribution in RhoA-mutant-inducible MDCK cells

(**a**, **b**) Serum-starved $RhoA^{V14}$-, (**c**, **d**) $RhoA^{V14/L40}$- or (**e**, **f**) $RhoA^{V14/C42}$-inducible MDCK cells were treated with (+; **b**, **d**, **f**) or without (−; **a**, **c**, **e**) 5 mM IPTG for 24 h. The cells were fixed and F-actin was stained with Alexa™ 488 phalloidin, as described in the Materials and methods section. The results shown are representative of three independent experiments that yielded similar results. The scale bar represents 50 μm.

indicating that, in addition to Rho-associated kinase, stress-fibre formation requires an additional effector, its interaction with RhoA being disrupted by mutation at 40 but not 42 in the effector loop. A similar finding with the same RhoA effector-loop mutants was reported in NIH 3T3 cells [23]. $RhoA^{V14/C42}$ was slightly less efficient in stress-fibre formation than $RhoA^{V14}$. Stress-fibre formation requires Rho-associated kinase and an additional effector. $RhoA^{V14/C42}$ shows potent GST-RBD-binding affinity comparable to $RhoA^{V14}$, suggesting that $RhoA^{V14}$ and $RhoA^{V14/C42}$ can activate Rho-associated kinase in the same level. Thus the mutation at 42 might reduce the interaction of RhoA with the additional effector. Recently, it was reported that mDia1 was a second target of Rho involved in stress-fibre formation [26,27]. It is plausible that $RhoA^{V14/C42}$ shows reduced affinity for mDia1 compared with $RhoA^{V14}$, and that the additional effector is mDia1. We next tried to clarify the effectors for RhoA-mediated regulation of tight-junction permeability. Rho-associated kinase was also involved in the RhoA-induced regulation of tight-junction permeability, because Y-27632 suppressed the RhoA-induced TER elevation. However, two effector-loop mutants, $RhoA^{V14/L40}$ and $RhoA^{V14/C42}$, failed to induce TER elevation, even though their interactions with Rho-

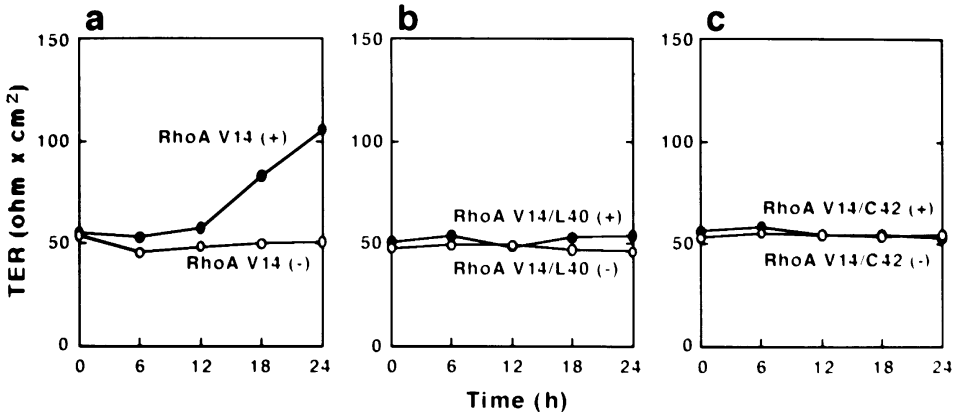

Figure 4 Effects of RhoA mutants on TER

Serum-starved (**a**) RhoA^{V14}-, (**b**) RhoA$^{V14/L40}$- or (**c**) RhoA$^{V14/C42}$-inducible MDCK cells were incubated in the presence (●) or absence (○) of 5 mM IPTG throughout the 24-h time course. The TER was monitored at the indicated times, and $\Omega \times cm^2$ were calculated as described in the Materials and methods section. The results shown are the means ± S.E.M. for triplicate determinations.

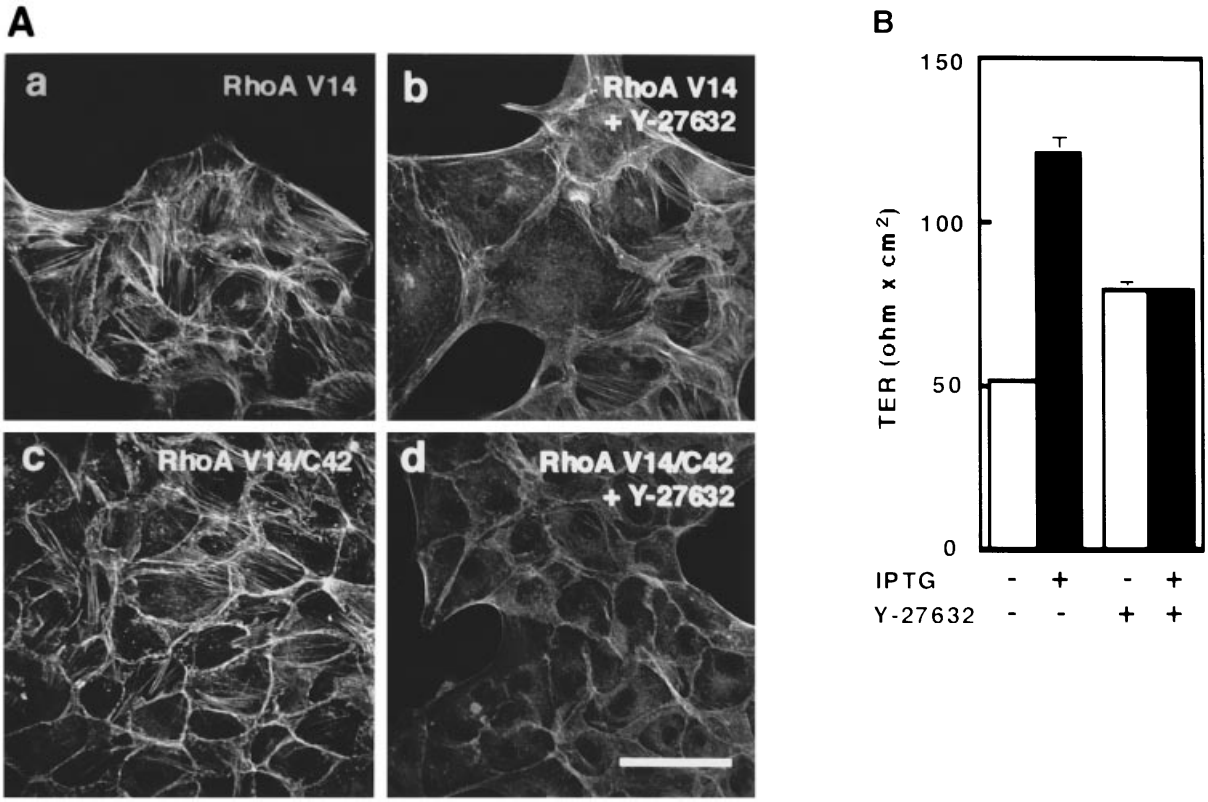

Figure 5 Effect of Rho-associated kinase inhibitor on RhoA-induced stress-fibre formation and elevation of TER

(**A**) Serum-starved RhoA^{V14}- or RhoA$^{V14/C42}$-inducible MDCK cells were incubated with 5 mM IPTG in the presence (**b** and **d**) or absence (**a** and **c**) of 10 μM Y-27632 for 24 h. The cells were fixed and F-actin was stained with Alexa^{TM} 488 phalloidin, as described in the Materials and methods section. (**B**) Serum-starved RhoA^{V14}- or RhoA$^{V14/C42}$-inducible MDCK cells were incubated for 18 h with vehicle, 5 mM IPTG, 10 μM Y-27632, or both. The TER was monitored and $\Omega \times cm^2$ were calculated as described in the Materials and methods section. The results shown are the means ± S.E.M. for triplicate determinations.

associated kinase were unimpaired, suggesting that an additional effector may be required for the RhoA-induced regulation of tight-junction permeability, and the interaction of RhoA with this additional effector is disrupted by mutation at either 40 or 42. Furthermore, the RhoA-induced TER elevation showed different sensitivity to effector-loop mutation from RhoA-induced stress-fibre formation. In view of this different sensitivity, RhoA-regulated barrier function and stress-fibre formation are mediated by different combinations of multiple effectors; that is, Rho-associated kinase is a common effector for the RhoA-

induced regulation of tight-junction permeability and stress-fibre formation, but these two RhoA functions require different additional effectors.

The tight junction is a regulated sealing barrier in the paracellular pathway [2]. Recent studies have identified several sealing proteins of tight junctions, occludin [28] and claudins [29], which are transmembrane proteins with four transmembrane domains and specifically localized to tight-junction domains. Occludin, in turn, is linked to peripheral membrane proteins, such as ZO-1 [30] and ZO-2 [31], participating in the formation of tight-junctional strands. Those juxtamembrane protein complexes are believed to be connected to and regulated by the peri-junctional filaments of actin and myosin [32]. It has been suggested that tension of the peri-junctional actin–myosin filaments influences solute permeation through tight junctions [33]. We have demonstrated here that Rho-associated kinase was involved in RhoA-regulated tight-junction permeability. Rho-associated kinase phosphorylates the myosin-binding subunit of myosin phosphatase and inactivates its activity [13], and also directly phosphorylates the myosin light chain [34], leading to the activation of myosin ATPase and producing contraction. Rho-associated kinase-induced contraction of the actin–myosin filaments may regulate the barrier function of the tight junction. Consonant with this view, antidiuretic hormone has been reported to induce phosphorylation of myosin light chain and thereby enhance solute permeability of tight junctions [35]. On the other hand, Y-27632 by itself increased basal TER levels in MDCK cells without expression of $RhoA^{v14}$, suggesting that Rho-associated kinase reduced TER in response to endogenous Rho. In contrast to the TER elevation induced by $RhoA^{v14}$, requiring a lag period after $RhoA^{v14}$ expression, the TER elevation by Y-27632 occurred within 1 h (results not shown). Considering these two effects of Y-27632, the inhibition of $RhoA^{v14}$-induced TER elevation by Y-27632 and Y-27632-induced TER elevation, Rho-associated kinase may show dual regulations of TER through somehow different mechanisms. Besides Rho-associated kinase, we suggested here that the RhoA-induced regulation of barrier function required another effector, which was sensitive to mutation at 40 or 42 in RhoA–effector interaction. In our recent study, we proposed the possibility that some gene expression was involved in the regulation of barrier function by the activation of Rho [19]. Requirement for a lag period in the TER elevation after the expression of $RhoA^{v14}$ supports this possibility as well. Rho has been known to regulate gene expression through activation of the transcription factor SRF (serum-response factor) [36]. Although Rho effectors responsible for SRF activation have not yet been unveiled, effector-loop mutations at 40 and 42 were reported to abrogate the RhoA-mediated activation of SRF [23,37]. Considering those observations, an effector, linking to activation of SRF, might participate in the RhoA-induced regulation of barrier function in concert with Rho-associated kinase.

In summary, we showed here that the RhoA-regulated barrier function of tight junctions is mediated by multiple effectors; Rho-associated kinase and some other as yet unknown effector(s). Tight junctions are well-organized structures but this regulation is very complex. This study will provide some clues to help to resolve this intricate complexity.

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