Simian Virus 40 Small Tumor Antigen Induces Deregulation of the Actin Cytoskeleton and Tight Junctions in Kidney Epithelial Cells

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There is increasing evidence that the transforming DNA tumor virus simian virus 40 (SV40) is associated with human malignancies. SV40 small tumor antigen (small t) interacts with endogenous serine/threonine protein phosphatase 2A (PP2A) and is required for the transforming activity of SV40 in epithelial cells of the lung and kidney. Here, we show that expression of SV40 small t in epithelial MDCK cells induces acute morphological changes and multilayering. Significantly, it also causes severe defects in the biogenesis and barrier properties of tight junctions (TJs) but does not prevent formation of adherens junctions. Small t-induced TJ defects are associated with a loss of PP2A from areas of cell-cell contact; altered distribution and reduced amounts of the TJ proteins ZO-1, occludin, and claudin-1; and marked disorganization of the actin cytoskeleton. Small t-mediated F-actin rearrangements encompass increased Rac-induced membrane ruffling and lamellipodia, Cdc42-initiated filopodia, and loss of Rho-dependent stress fibers. Indeed, these F-actin changes coincide with elevated levels of Rac1 and Cdc42 and decreased amounts of RhoA in small t-expressing cells. Notably, these cellular effects of small t are dependent on its interaction with endogenous PP2A. Thus, our findings provide the first evidence that, in polarized epithelial cells, expression of small t alone is sufficient to induce deregulation of Rho GTPases, F-actin, and intercellular adhesion, through interaction with endogenous PP2A. Because defects in the actin cytoskeleton and TJ disruption have been linked to loss of cell polarity and tumor invasiveness, their deregulation by PP2A and small t likely contributes to the role of SV40 in epithelial cell transformation.

A majority of human tumors arise from epithelial cells. Invasion is characterized by loss of polarity, deregulation of cell adhesion, and cytoskeletal disruption. There is increasing evidence that the transforming DNA tumor virus simian virus 40 (SV40) is associated with human malignancies (17, 33). Inactivation of retinoblastoma proteins following expression of the SV40 large T antigen in MDCK cells results in an epithelial-mesenchymal conversion and invasiveness (21). Studies with transgenic mice have shown that SV40 small tumor antigen (small t) cooperates with the large T antigen in inducing tumors in slowly dividing epithelial cells of the lung and kidney. Small t is also absolutely required for SV40-mediated formation of lung and kidney adenocarcinoma (2). Moreover, it promotes serum-independent cell growth in mammary gland epithelial cells and induces breast cancer formation in transgenic mice (9).

Following SV40 infection (23, 30) or ectopic expression (35), small t specifically associates with endogenous serine/threonine protein phosphatase 2A (PP2A). PP2A is believed to exist in vivo predominantly as a heterotrimeric complex comprising a core enzyme containing the catalytic C and structural A subunits, which is bound to a regulatory B subunit. Significantly, distinct regulatory B subunits mediate PP2A functional specificity by controlling the intracellular targeting and modulating the catalytic activity and substrate specificity of the enzyme (reviewed in reference 34). Remarkably, small t forms a complex with endogenous AC dimers either directly or indirectly by displacing the Bα subunit from corresponding ABαC holoenzymes (20, 26, 29, 35). Formation of AC-small t complexes results in dramatic deregulation of PP2A activity and functional specificity (20, 35, 36, 37). It has been shown that interaction of small t with PP2A is required for the transformation of human epithelial cells coexpressing oncogenic HA-Ras and the gene encoding the human telomerase catalytic subunit (12). However, not much is known about the ability of small t alone to modulate specific epithelial properties. We have recently shown that ABαC, a major PP2A holoenzyme, is targeted to the apical membrane domain of well-polarized MDCK cells, where it interacts with and regulates the tight junction (TJ) proteins ZO-1, occludin, and claudin-1 (24). Importantly, TJs play a critical role in maintaining the permeability barrier and polarity of epithelia (1). Together, these observations prompted us to undertake a detailed analysis of the effects of small t in polarized MDCK cells. We show here that deregulation of endogenous PP2A following expression of small t is sufficient by itself to induce alterations in the actin cytoskeleton and deregulation of TJs in these cells.

MATERIALS AND METHODS

Antibodies and plasmids. Antibodies used in this study included mouse anti-SV40 small t (Ab-3) (Oncogene Research Products) and Pab419 (35); rabbit anti-A (36); rabbit anti-B (24); mouse anti-C and mouse anti-E-cadherin (Transduction Laboratories); rat anti-ZO-1 (Chemicon International Inc.); rabbit anti-claudin-1 and rabbit anti-occludin (Zymed); rabbit anti-Rac1, rabbit anti-Cdc42, and mouse anti-RhoA (Cytoskeleton Inc.); and affinity-purified fluorescein isothiocyanate (FITC)- or Texas red-coupled secondary antibodies and FITC-labeled phalloidin (Molecular Probes Inc.). Expression vectors encoding SV40 small t and small t mutant 3 have been described previously (35, 37).
Cell culture and characterization of stable MDCK cell lines. Madin-Darby canine kidney (MDCK) strain II D3 cells were cultured in Dulbecco modified Eagle medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS) (HyClone), and plated on Transwell filters (Costar) to induce polarization (24). Cells were transfected with Lipofectamine Plus reagent (Invitrogen). Stable colonies were selected with 800 μg of Geneticin (Invitrogen) per ml. The expression levels of transfected proteins were constantly monitored by immunofluorescence and immunoblotting, and seven distinct stably transfected small t cell populations were used throughout our studies. For all of the studies described here, similar results were found for cloned and pooled stable cell populations. MDCK cells stably transfected with the corresponding empty vector were used as control cells and behaved like nontransfected cells in our experiments. Sodium butyrate (5 mM) (Sigma) was added in the cell culture medium for ~16 h prior each experiment to enhance expression of transfected proteins (24).

Calcium switch experiments. For the studies of the role of small t in TJ assembly, cells were serum and Ca2+-starved by overnight incubation in low-calcium (LC) medium (Ca2+-free minimum essential medium; Invitrogen) containing 1% dialyzed FBS and then transferred to normal-Ca2+ (NC) medium (DMEM plus 10% FBS) (24).

Phase-contrast microscopy. The cell medium was replaced with phenol red-free DMEM (Invitrogen) and examined with a 20x phase-contrast filter on a Zeiss microscope equipped with a digital camera. Images were acquired with NIH image software and transferred to Adobe Photoshop 5.5 for printing.

Confocal microscopy. Confocal cells grown on Transwell filters or on glass coverslips were fixed with methanol for 5 min at −20°C (for TJ proteins and PP2A subunits), and labeled sequentially for 1 h with primary and secondary antibodies (1:200) (24). For the visualization of F-actin and small t, cells were fixed for 20 min with 4% paraformaldehyde and permeabilized for 5 min with 0.1% Triton X-100 prior to staining. F-actin was detected by using fluorescent phalloidin. The samples were mounted with Fluoromount-G (Fisher) and examined on a Leica TCS SP confocal microscope with a 63x objective. Images (x-y or x-z sections across cells) were directly captured, saved, and transferred to Adobe Photoshop 5.5 for printing. The specificity of the labeling was verified by omitting first or second antibodies during the staining procedures.

Cell fractionation, immunoprecipitation, and Western blotting. Total extracts, NP-40 detergent-soluble and -insoluble fractions, or cytosolic and membrane fractions were prepared from the cells exactly as described previously (24). Equivalent amounts of proteins from the fractions, as determined with the Bio-Rad protein assay kit (Bio-Rad), were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5% (for ZO-1), 8% (for occludin and E-cadherin), 10% (for claudin-1 and PP2A subunits), and 12% (for small t and small G proteins) polyacrylamide gels and analyzed by immunoblotting. Immunoreactive proteins were detected with SuperSignal chemiluminescent substrates (Pierce).

Immunoprecipitation assays were carried out exactly as described previously (24). Equivalent amounts of proteins from the fractions, as determined with the Bio-Rad protein assay kit (Invitrogen), were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5% for ZO-1, 8% for occludin and E-cadherin, 10% for claudin-1 and PP2A subunits, and 12% (for small t and small G proteins) polyacrylamide gels and analyzed by immunoblotting. Immunoreactive proteins were detected with SuperSignal chemiluminescent substrates (Pierce).

Measurement of TER and paracellular diffusion of nonionic molecular tracers. For measurement of transepithelial resistance (TER) and paracellular diffusion of nonionic molecular tracers, cells were plated at confluence and grown in 24-well plates for 3 days. These experiments were performed under conditions that prevented small t-induced cell multilayering, i.e., by using only MDCK-small t clones expressing low basal levels of SV40 small t. Expression of high levels of small t was induced by incubating monolayers with sodium butyrate for 16 h prior to the TER and tracer flux measurements. TER values were measured in duplicate wells an Endohm volt ohmmeter (World Precision Instruments), exactly as described previously (24). TJ leakiness was assessed by measuring the diffusion of 3HJulin (Amersham Pharmacia Biotech) and 3HTmannitol (NEN Life Science Products, Inc.) across the membrane, exactly as reported previously (24).

RESULTS

Expression of SV40 small t induces morphological defects and multilayering in polarized MDCK cells. Several distinct populations of MDCK cells stably expressing SV40 small t (MDCK-small t cells) were generated to investigate the potential role of this tumor antigen in the deregulation of epithelial cell properties. As illustrated in Fig. 1, expression of SV40 small t dramatically altered the phenotype of MDCK cells. Growing parental MDCK cells stably transfected with the empty vector alone (control MDCK cells) formed characteristic small, compact cell islets. In stark contrast, MDCK-small t cells grew as disorganized colonies of spreading, large, flat cells with an irregular, sometimes elongated fibroblastic-like shape and partially overlapping extensions. At confluency and post-confluency, control MDCK cells formed tight monolayers with a typical cobblestone-like appearance. On the other hand, confluent MDCK-small t monolayers displayed irregularities in the areas of cell-cell contact, including the presence of large intercellular gaps and indistinguishable cell borders. Moreover, MDCK-small t cells rapidly started to pile up soon after reaching confluency, resulting in multilayering. Notably, a significant amount of huge, multinucleated cells was found in all MDCK-small t cell cultures that were generated, with their proportion increasing with time in culture.

Immunofluorescence analysis of confluent MDCK-small t monolayers grown on glass coverslips revealed that the tumor antigen was absent from the plasma membrane but localized primarily in the cytoplasm and/or nuclei of the cells (Fig. 2A), as described previously for other cell types (4, 35). In addition, multiple nuclei immunoreactive with anti-small t antibody could be observed in each of the numerous giant cells present in the culture. Confluent MDCK-small t cells were also grown for 5 days on Transwell filters, which is known to induce the formation of polarized MDCK monolayers. Confocal images of x-y sections (Fig. 2B) or transversal (x-z) sections (Fig. 2C) performed directly across the cells further confirmed that MDCK-small t cells abnormally grew as multiple layers. The x-z views of the cells also established that small t was not present at the plasma membrane but was distributed across the cytoplasm and localized in the nucleus.

PP2A is redistributed in small t-expressing cells. We have previously reported that a pool of the AβC heterotrimer localizes at the apical membrane of polarized MDCK cells (24). Accordingly, antibodies directed against the A, Bα, or C subunit of PP2A clearly labeled areas of cell-cell contact in polarized control cells (Fig. 3A). However, in MDCK-small t cells grown on filters, these PP2A subunits were essentially absent from the membrane; instead, they were diffusely redistributed in the cytoplasm and/or nuclei. Moreover, the immunofluorescent signal for Bα was somewhat weak in MDCK-small t cells, in agreement with our previous studies showing decreased expression of Bα in small t-expressing CV-1 cells (35). These reduced cellular amounts of Bα subunits probably result from their down-regulation following their displacement by transfected small t proteins from corresponding AβC heterotrimers (35).

Our immunofluorescence data indicate that the distribution of the A and C subunits overlaps with that of small t in MDCK-small t cells. Indeed, as expected from our previous studies (35), expressed small t formed a complex with endogenous PP2A in stably transfected MDCK cells, as judged by the presence of both the A and catalytic C subunits of PP2A in immunoprecipitations performed with monoclonal anti-small t antibody (Fig. 3B).
Expression of SV40 small t inhibits TJ assembly. The results shown in Fig. 3A clearly indicate that small t induces significant changes in the intracellular subunit composition and distribution of PP2A in MDCK cells. Given the observations that PP2A is a key intracellular target of small t (34) and critically modulates TJs (24), we next addressed the hypothesis that the formation of PP2A-small t complexes elicits TJ deregulation. First, to investigate the potential role of small t in TJ assembly, control and MDCK-small t cells were comparatively analyzed by confocal microscopy for the distribution of three major TJ proteins, i.e., ZO-1, occludin, and claudin-1, during Ca^{2+} switch experiments (Fig. 4). The essential role of calcium in the formation of intercellular junctions is well established (3). Cells were Ca^{2+} starved overnight to induce complete disruption of intercellular junctions, resulting in internalization and redistribution of TJ proteins from the cell periphery to the cytosol. The biogenesis of functional junctional complexes was then triggered upon transferring cells cultured in LC medium to NC medium (10). As expected from earlier reports (6, 24, 38, 40), the Ca^{2+} switch initiated a rapid sorting of ZO-1, occludin, and claudin-1 from the cytosol to the membrane in control MDCK cells, although significant pools of cytosolic TJ proteins were still present 2 h after the Ca^{2+} switch. The complete TJ stabilization and resealing, as measured by restoration of the TER, was achieved 24 h after the Ca^{2+} switch (3). It correlated with the full redistribution of TJ proteins to cell-cell contact sites, as described previously (24, 38). In contrast to the case for control cells, expression of small t significantly delayed the redistribution of TJ proteins to the cell periphery. Even 24 h after the Ca^{2+} switch, cytosolic pools of these TJ proteins were still present in small t-expressing MDCK cells.
FIG. 2. Analysis of SV40 small t distribution by confocal microscopy in stably transfected MDCK cells. MDCK-small t cells were grown on Transwell filters in NC medium and analyzed by confocal microscopy with monoclonal anti-small t antibodies. Bars, 60 μm. (A) Representative images of two separate small t populations are shown in the left and middle panels. A representative multinucleated cell is enlarged in the right panel; the arrow indicates the absence of small t from the plasma membrane. (B) Thirty-two x-y sections were performed across the cells; successive sections 11, 16, 22, and 30 are shown. (C) Representative transversal x-z view of the cells. The double arrow indicates the thickness of the normal MDCK monolayer.

FIG. 3. Analysis of PP2A subunit distribution in MDCK-small t cells. (A) Control and MDCK-small t cells were grown on Transwell filters in NC medium and analyzed by confocal microscopy with anti-A, anti-Bα, and anti-C subunit antibodies. Bars, 10 μm. Note that under our experimental conditions, the monoclonal anti-C antibody used here failed to recognize nuclear PP2A, in contrast to the anti-A antiserum. (B) Total cell lysates were prepared from control MDCK or MDCK-small t cells cultured in NC medium, immunoprecipitated with anti-small t antibodies, and analyzed by immunoblotting for the presence of small t and the A and C subunits of PP2A.
FIG. 4. Effects of SV40 small t on the distribution of junctional proteins during TJ biogenesis. Confluent control and MDCK-small t cells were Ca\(^{2+}\) starved overnight in LC medium to induce complete TJ disruption and then switched for the indicated times to NC medium to induce TJ assembly. Cells were analyzed by confocal microscopy for the distribution of ZO-1, occludin, claudin-1, and E-cadherin (E-cad.). Bars, 10 μm.

FIG. 5. Expression of SV40 small t inhibits TJ assembly. (A and B) Confluent control and MDCK-small t cells were Ca\(^{2+}\) starved overnight in LC medium and then switched to NC medium. (A) Equivalent amounts of proteins (~40 μg) from detergent-soluble (lanes S) and -insoluble (lanes I) fractions were prepared from the cells 5 or 24 h after the Ca\(^{2+}\) switch and analyzed by Western blotting for the presence of junctional proteins. (B) Equivalent amounts of proteins (~50 μg) from cytosolic (lanes Cy) and membrane (lanes M) fractions were prepared from the cells 24 h after the Ca\(^{2+}\) switch and analyzed by Western blotting for the presence of ZO-1, small t, and PP2A subunits. (B) Sodium butyrate + or − was added to the culture medium of MDCK cells in the absence of small t expression. Results are expressed as the percentage of the relative initial resistance (TER = 1,300 ± 100 Ω·cm\(^2\)) measured prior to the Ca\(^{2+}\) switch in noninduced cells cultured in NC medium. Values are the means ± standard deviations of duplicate determinations performed in four separate experiments with four distinct populations of transfectants. The representative immunoblot in the upper panel shows the total expression levels of small t in the cells before (lane −) and after (lane +) treatment with sodium butyrate.
proteins were still predominant in MDCK-small t cells. Furthermore, the membrane staining for ZO-1, occludin, and claudin-1 was strikingly uneven and discontinuous in these cells, in stark contrast with the characteristic "chicken wire" appearance of these proteins in controls. Although expression of small t noticeably inhibited the membrane redistribution of TJ proteins, it did not appear, under identical experimental conditions, to appreciably prevent the sorting of the adherens junction protein E-cadherin from the cytosol to the membrane during Ca\(^{2+}\)-induced junctional assembly.

Detergent insolubility is a commonly used indicator of the cytoskeletal association and incorporation of proteins into large junctional complexes in MDCK cells (38). Accordingly, newly formed TJ protein complexes become resistant to non-

FIG. 6. Expression of SV40 small t induces TJ defects in MDCK cells cultured in NC medium. Cells were grown for 3 days on Transwell filters in NC medium. (A) Control cells and two separate populations of small t-expressing MDCK cells were analyzed by confocal microscopy for the distribution of junctional proteins. Bars, 10 μm. (B) Equivalent amounts of proteins (−30 μg) from total lysates prepared from control (lane C) and small t-expressing (lane St) cells were analyzed by Western blotting for the presence of junctional proteins. Note the decreased levels of TJ proteins in small t-expressing cells. In particular, the upper band corresponding to slow-migrating, phosphorylated occludin species present in control cell lysates is nearly undetectable in extracts prepared from MDCK-small t cells. (C) Paracellular diffusion of \(^{[3]H}\)mannitol and \(^{[3]H}\)inulin was measured before (noninduced) and after (induced) preincubation of subsets of MDCK-small t cells with sodium butyrate, as described for Fig. 5C. Results are expressed as the percentage of tracer flux measured in noninduced cells and are the means ± standard deviations of triplicate determinations performed in four distinct experiments with four separate cell populations.
ionic detergent extraction during TJ assembly (24, 38). To further assess whether expression of small t affects the association of ZO-1, occludin, and claudin-1 with TJs, we compared by immunoblotting their distribution in detergent-soluble and -insoluble fractions prepared from control MDCK and MDCK-small t cells during Ca\textsuperscript{2+} switch experiments (Fig. 5A). TJ assembly was induced by switching MDCK cells from LC to NC medium and correlated with the progressive appearance of detergent-insoluble, phosphorylated TJ proteins, including slow-migrating, phosphorylated occludin species (24, 40). Notably, expression of small t largely prevented the accumulation of phosphorylated, detergent-insoluble TJ proteins during TJ assembly, in agreement with the results of our immunofluorescence studies indicating that small t inhibits the redistribution of TJ proteins at the membrane. On the other hand, it did not affect the recruitment of E-cadherin to detergent-insoluble fractions during junctional biogenesis, although it slightly increased the proportion of detergent-soluble E-cadherin relative to that in control cells.

Since a pool of Aboc is recruited to the membrane during TJ formation (24), we next compared the distributions of PPP2A subunits, small t, and ZO-1 in cytosolic and membrane fractions prepared from control and MDCK-small t cells 24 h after the Ca\textsuperscript{2+} switch (Fig. 5B). Like that of ZO-1, the accumulation of Aboc at the membrane during TJ biogenesis was dramatically inhibited following small t expression. Moreover, in accordance with the findings in our immunofluorescence studies, small t was not detected in the membrane fraction, and the cellular levels of Ba were greatly reduced in MDCK-small t cells relative to control cells.

TJ assembly also correlates with the development of TER in MDCK cells. Because differences in the cell density between stable control and MDCK-small t cells were unavoidable and substantial, directly comparing their TER values would not be very meaningful. Moreover, elevated amounts of expressed small t antigen were associated with morphological changes and progressive cell multilayering, which impede the interpretation of such experiments. To circumvent these problems, we used stable MDCK-small t monolayers expressing low basal levels of small t. The development of TER was examined by switching cells from LC to NC medium before and after a 16-h preincubation with sodium butyrate (Fig. 5C). This incubation time was sufficient to dramatically enhance expression of small t in these cells at the start of the experiment; at the same time, it was not long enough to allow for the development of acute changes in cell density and shape that occur following prolonged and heightened expression of the antigen. MDCK-small t cells treated with sodium butyrate developed TER with much slower kinetics than untreated cells. Under identical experimental conditions, treatment with sodium butyrate did not affect the TER of MDCK cells (24).

Thus, together, these findings indicate that ectopic expression of small t inhibits TJ assembly.

Expression of SV40 small t induces alterations in the cellular levels and distribution of TJ proteins and TJ leakiness. We next investigated the effects of expressing small t on the intracellular localization of junctional proteins in confluent MDCK cells cultured on Transwell filters in NC medium, which normally allows for complete cell polarization and mature TJ formation (Fig. 6). Immunofluorescence analysis of polarized control cells revealed the characteristically chicken wire appearance of the junctional protein staining at the membrane. In contrast, expression of small t induced profound alterations in the normal distribution pattern of ZO-1, occludin, and claudin-1, including interrupted, twisted, and beaded lines in the regions of cell-cell contact, together with diffuse cytoplasmic staining. Moreover, while the cell shape of parental control MDCK cells was very regular, MDCK-small t cells displayed remarkable irregularities in their cell shape and size, as observed during phase-contrast microscopy (Fig. 1) and TJ assembly (Fig. 4). Notably, these abnormalities in the distribution of TJ proteins were found in most cells, regardless of the nature of small t-induced morphological changes. They were also observed during transient transfection of MDCK cells (not shown). However, it is important to point out that their extent was directly proportional to the total cellular amounts of expressed small proteins (not shown). Unlike that for TJ proteins, the staining for E-cadherin at the cell-cell contact sites appeared to be retained to a large extent following expression of small t. However, residual cytoplasmic E-cadherin pools were sometimes observed in multilayered MDCK-small t cells. In addition, somewhat diffuse and poorly demarcated E-cadherin staining occasionally occurred at sites of acute TJ disruption. Interestingly, comparative analysis of total cell lysates indicated that expression of small t was also associated with a dramatic decrease in the cellular amounts of ZO-1, occludin, and claudin-1 but not E-cadherin (Fig. 6B).

We next assessed whether the defects in the cellular levels and distribution of TJ proteins correlated with changes in the paracellular permeability of MDCK-small t cells. The paracellular diffusion of \[^{[3]}H\]mannitol and \[^{[3]}H\]inulin was carefully measured across subsets of MDCK-small t monolayers before and after preincubation with sodium butyrate, following the specific guidelines and experimental conditions described for TER analyses. As illustrated in Fig. 6C, the relative radioactive tracer fluxes increased by up to 5.5-fold after induction of small t expression, indicating that this viral antigen is capable of eliciting TJ leakiness.

The actin cytoskeleton is disorganized in MDCK-small t cells. There is clear evidence that numerous TJ proteins can directly interact with cortical actin filaments at TJs, either directly or indirectly through the scaffolding protein ZO-1 (39). It is also well recognized that the complete reestablishment of the architecture of the actin cytoskeleton plays a crucial role during Ca\textsuperscript{2+}-mediated junctional biogenesis (3). Since small t has been shown to disorganize actin filaments in rat cells (11), we addressed the hypothesis that disruption of F-actin contributes to the inhibitory effects of small t on TJ assembly. F-actin patterns in control and MDCK-small t cells were visualized by confocal microscopy after labeling with fluorescent phalloidin (Fig. 7). In control cells, the recruitment of actin to a ring-like structure occurred rapidly during TJ assembly. When confluent cells were cultured in NC medium, the classical perijunctional F-actin staining pattern was observed at the apical membrane, while bundles of stress fibers were present at the basal section of the monolayers, as reported previously (18, 28). In contrast, the overall organization of the F-actin network was highly disrupted in MDCK-small t cells subjected to the Ca\textsuperscript{2+} switch or cultured in NC medium. Defects in the arrangement of F-actin in these cells encompassed increased membrane edge...
ruffling, an intense loss of basal stress fibers, and formation of thick actin aggregates in the plane of the apicolateral membrane. As observed by phase-contrast microscopy, actin labeling of MDCK-small t cells also revealed their highly irregular

FIG. 7. Expression of SV40 small t in MDCK cells induces the reorganization of the actin cytoskeleton. (A) Confluent control and MDCK-small t cells were Ca^{2+} starved overnight in LC medium and then switched for 2 h from LC to NC medium. (B) Confluent control and MDCK-small t cells were grown in NC medium. For both panels, cells were fixed and analyzed by confocal microscopy for the distribution of F-actin by using FITC-labeled phalloidin. Representative apical and basal x-y sections are shown for cells cultured in NC medium (B). Bars, 10 μm.

medium, switched for 2 h from LC to NC medium, and double labeled with mouse anti-small t and FITC-phalloidin. (B) F-actin distribution at the apical and basal sections of the monolayer. (C) Polarized cells were analyzed for the distribution of Bα, E-cadherin, occludin, and ZO-1. The staining pattern of claudin-1 and of PP2A A and C subunits in MDCK-small t\textsuperscript{mut3} cells was similar to that in control cells (not shown). (D) Equivalent amounts of proteins (~30 μg) from total lysates prepared from control and MDCK-small t\textsuperscript{mut3} cells were analyzed by immunoblotting for the presence of TJ proteins.

FIG. 8. Analysis of MDCK cells expressing small t mutant 3. (A to C) MDCK-small t\textsuperscript{mut3} cells expressing a PP2A binding-defective small t mutant were grown in NC medium and analyzed by confocal microscopy. Bars, 10 μm. (A) Cells were Ca^{2+} starved overnight in LC


A Small t mut3 F-actin

B Actin, apical Actin, basal

C Bα E-cadherin

ZO-1 Occludin

D ZO-1 Control Small t Mut3 Occludin Claudin-1
cell shape with poorly demarcated cell boundaries and extensive interdigitations and lamellipodia.

The effects of small t on TJs and F-actin are dependent on its interaction with PP2A. To confirm that the cellular effects of small t are mediated through its interaction with PP2A, we also examined whether expressing a small t mutant (small t mutant 3) defective in PP2A binding (35) would affect the organization of F-actin and the distribution of PP2A and junctional proteins in MDCK cells. As with the wild-type small t, expressed small t mutant proteins were localized in the cytoplasm and/or nuclei of cells (Fig. 8A). However, in contrast to wild-type small t, they failed to induce any morphological changes and F-actin rearrangements in cells subjected to a Ca\(^{2+}\) switch (Fig. 8A) or cultured in NC medium (Fig. 8B), relative to controls. The distribution of PP2A and TJ proteins (Fig. 8C) and the cellular expression levels and phosphorylation of TJ proteins (Fig. 8D) also appeared to be unaltered in MDCK cells following expression of small t mutant 3. Thus, the deregulation of the actin cytoskeleton and intercellular adhesion induced by small t is PP2A dependent.

Rho GTPases are deregulated in MDCK-small t cells. In fibroblasts, activation of the Rho GTPases RhoA, Rac1, and Cdc42 has been linked to the formation of specific actin membrane structures, namely, stress fibers and focal adhesions (RhoA), lamellipodia and membrane ruffles (Rac1), and filopodia (Cdc42) (reviewed in reference 13). It has been shown that activation of these small G proteins produces similar effects in MDCK cells cultured at low cell densities (18). We thus investigated whether these Rho GTPases were involved in the reorganization of F-actin by small t (Fig. 9). MDCK cells expressing wild-type (MDCK-small t) or mutant (MDCK-small t\(^\text{mutant3}\)) small t proteins were grown at low density in NC medium and then serum starved overnight. We generally observed that the patterns of cortical actin were similar in serum-starved and nonstarved cells (not shown). However, stress fibers were much less abundant in starved MDCK-small t\(^\text{mutant3}\) cells than in cells cultured in NC medium; they were virtually absent in starved MDCK-small t cells (not shown). Next, specific RhoA-, Rac1-, or Cdc42-initiated changes in F-actin patterns were induced in the starved cells upon stimulation with selective extracellular factors (reviewed in reference 13). Incubation with epidermal growth factor (EGF) resulted in a marked increase in membrane edge ruffling and stress fibers in MDCK-small t\(^\text{mutant3}\) cells (Fig. 9A). However, stress fibers were not restored in EGF-stimulated MDCK-small t cells; instead, intense membrane ruffling and a strikingly beaded appearance of the cortical actin were noticeable. This Rac1-initiated membrane ruffling activity was more readily observed at the sites of cell-cell contacts created by coiling migratory cells than in isolated cells, as reported previously (18). Both MDCK-small t\(^\text{mutant3}\) and MDCK-small t cells developed cdc42-initiated filopodia in response to bradykinin, but this effect was overly magnified in MDCK-small t cells (Fig. 9B). As expected, numerous bundles of stress fibers were present in MDCK-small t\(^\text{mutant3}\) cells following stimulation with lysophosphatidic acid (LPA), which is known to activate RhoA-dependent signaling pathways (Fig. 9C). In stark contrast, some lamellipodia and filopodia but very few, if any, stress fibers were visible in LPA-stimulated MDCK-small t cells.

The membrane ruffling, increased filopodia, and loss of stress fibers induced by small t are indicative of Rac1 and cdc42 activation and RhoA inhibition. We thus attempted to assess the effect of expressed small t on the activity of these Rho-GTPases by using specific pull-down assays coupled with Western blotting (RhoA, Rac1, and cdc42 activation assay kits [Cytoskeleton Inc.]). Unfortunately, due to the low sensitivity of such assays and transient nature of small G protein activation, we were unable to successfully detect activated Rho GTPases in our cellular samples under our experimental conditions. However, Western blot analysis of total cell extracts revealed that the levels of Rac1 and Cdc42 were considerably increased, whereas those of RhoA were reduced, in MDCK-small t cells compared to control and MDCK-small t\(^\text{mutant3}\) cells (Fig. 9D). Together, these data suggest that expression of small t in MDCK cells induces a PP2A-dependent deregulation of Rho GTPases.

**DISCUSSION**

We show here that expression of small t in MDCK cells induces marked cell morphological changes. The observation that multinucleated cells accumulate in MDCK-small t cultures is reminiscent of earlier studies showing that CV-1 cells (E. Sontag and M. Mumby, FASEB J., vol. 8, abstr. A1235, 1994) and human fibroblasts (8) overexpressing small t become multinucleated as a result of defects in cytokinesis. Small t is known to cooperate with large T antigen to drive cell proliferation and transformation (reviewed in reference 31). Recently, it has been reported that small t is required for the transformation of human epithelial cells in the presence of oncogenic HA-

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FIG. 9. Analysis of the role of expressed wild-type and mutant small t proteins in the regulation of Rho GTPases and F-actin rearrangements in MDCK cells. Control, MDCK-small t (Wt Small t), and MDCK-small tmutant (Small t mut3) cells were cultured at low densities in NC medium and serum starved overnight by incubation in DMEM containing 0.2% dialyzed FBS (HyClone). (A to C) Serum-starved cells were stimulated for 10 min with either EGF (50 ng/ml; Upstate Biotechnology) (A), bradykinin (1 μM; Sigma) (B), or LPA (10 μM; Sigma) (C). Cells were then labeled with FITC-phalloidin and analyzed by immunofluorescence microscopy for the distribution of F-actin. Bars, 10 μm (A) and 5 μm (B and C). Note that F-actin patterns in MDCK-small tmutant and control cells (not shown) were indistinguishable. (D) Equivalent amounts of proteins (~100 μg) from total cell lysates were simultaneously analyzed by Western blotting for the expression levels of RhoA, Rac1, and Cdc42. A representative blot is shown; similar results were found in two other experiments.
to the transforming activity of small t (27). In any case, the results presented there strongly support a crucial role for small G protein-initiated F-actin rearrangements in the deregulation of epithelial cell adhesion by small t. Importantly, the balance between Rho, Rac, and cdc42 activities is critical for maintaining MDCK cell polarity (reviewed in references 5 and 32). Indeed, changes in the activity and/or overexpression of Rho family GTPases have been linked to malignant transformation of epithelial cells (7, 16). Likewise, there is ample evidence that deregulation of TJ is associated with cancer metastasis (reviewed in reference 22). In conclusion, deregulation of the activity and cellular levels of Rho GTPases, disorganization of F-actin, and disruption of TJ functions are likely to be key events in epithelial cell transformation by small t/PP2A.

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REFERENCES