Constitutively Active Mutant of the Mitogen-activated Protein Kinase Kinase MEK1 Induces Epithelial Dedifferentiation and Growth Inhibition in Madin-Darby Canine Kidney-C7 Cells*

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Overexpression of a constitutively active mitogen-activated protein kinase kinase (MAPKK or MEK) induces neuronal differentiation in adrenal pheochromocytoma 12 cells but transformation in fibroblasts. In the present study, we used a constitutively active MAPK/extracellular signal-regulated kinase (ERK) kinase 1 (MEK1) mutant to investigate the function of the highly conserved MEK1-ERK2 signaling module in renal epithelial cell differentiation and proliferation. Stable expression of constitutively active MEK1 (CA-MEK1) in epithelial MDCK-C7 cells led to an increased basal and serum-stimulated ERK1 and ERK2 phosphorylation as well as ERK2 activation when compared with mock-transfected cells. In both mock-transfected and CA-MEK1-transfected MDCK-C7 cells, basal and serum-stimulated ERK1 and ERK2 phosphorylation was almost abolished by the synthetic MEK inhibitor PD098059. Increased ERK2 activation due to stable expression of CA-MEK1 in MDCK-C7 cells was associated with epithelial dedifferentiation as shown by both a dramatic alteration in cell morphology and an abolished cytokeratin expression but increased vimentin expression. In addition, we obtained a delayed and reduced serum-stimulated cell proliferation in CA-MEK1-transfected cells (4.6-fold increase in cell number/cm² after 5 days of serum stimulation) as compared with mock-transfected controls (12.9-fold increase in cell number/cm² after 5 days). This result was confirmed by flow cytometric DNA analysis showing that stable expression of CA-MEK1 decreased the proportion of MDCK-C7 cells moving from G0/G1 to G2/M as compared with both untransfected and mock-transfected cells. Taken together, our data demonstrate an association of increased basal and serum-stimulated activity of the MEK1-ERK2 signaling module with epithelial dedifferentiation and growth inhibition in MDCK-C7 cells. Thus, the MEK1-ERK2 signaling pathway could act as a negative regulator of epithelial differentiation thereby leading to an attenuation of MDCK-C7 cell proliferation.

Extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2)† represent one subfamily of serine/threonine protein kinases collectively referred to as the mitogen-activated protein kinase (MAPK) family. They have the unique feature of being activated by phosphorylation on threonine and tyrosine residues by an upstream dual-specificity kinase called MAPK kinase (MAPKK or MKK) or MAPK/ERK kinase (MEK) (reviewed in Refs. 1 and 2). The MEKs upstream of ERKs constitute an evolutionarily conserved family of protein kinases that includes at least three highly homologous mammalian isoforms, namely MEK1a, MEK1b, and MEK2 (2). They are highly specific for both of their downstream targets ERK1 and ERK2 (2) and are typically activated by serine/threonine phosphorylation catalyzed by three different classes of upstream kinases: the Raf family of serine/threonine kinases, Raf-1, A-Raf, and B-Raf (3–7), the protooncogene product Mos (8, 9), and the MEK kinase 1 (MEKK1) (10). Despite their high degree of similarity, MEK1a and MEK2 are somewhat different in their ability to phosphorylate their substrates, ERK1 and ERK2 (11). However, although different MEK isoforms were previously shown to be differentially regulated, they appear to elicit similar transcriptional as well as morphological responses and to share ERK2 as a downstream effector (12).

A MAPK kinase (MKK or MEK) recently cloned from Chinese hamster lung fibroblasts (13) encodes a protein of 45 kDa that exhibits a high identity with the Xenopus (14) and murine (15) amino acid sequences. The high level of identity indicated that it represents the Chinese hamster homologue of the human isoform MKK1 or MEK1 (16, 17). Constitutively active as well as dominant negative MEK mutants have been utilized by different laboratories to study the function of the highly conserved MEK-ERK module in cell proliferation and differentiation. Expression of constitutively active MEK mutants, generated by substitution of the Raf1-dependent regulatory phosphorylation sites serine 218 and serine 222 by aspartic acid or glutamic acid, has been reported to transform fibroblasts and to induce tumor formation in nude mice (18–20). Moreover, overexpression of a S222E mutant of MEK, which showed enhanced MEK activity as well as stimulation of ERK and p90Rsk, increased proliferation and altered morphology of NIH 3T3 fibroblasts but failed to induce their growth in soft agar (21). In contrast to these experiments performed in fibroblasts, a constitutively active MEK1 mutant stimulated PC12 cell differentiation, while interfering mutants of MEK1 inhibited ligand-induced neurite outgrowth (19). Thus, constitutive activation of the only known ERK activator MEK leads to transformation of fibroblasts but differentiation of PC12 cells, myelin basic protein; MDCK, Madin-Darby canine kidney; PC12, adrenal pheochromocytoma 12; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CA, constitutively active; FACS, fluorescence-activated cell sorter; MEM, minimal essential medium; HA, hemagglutinin.

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‡ The abbreviations used are: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; MBP, myelin basic protein; MDCK, Madin-Darby canine kidney; PC12, adrenal pheochromocytoma 12; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CA, constitutively active; FACS, fluorescence-activated cell sorter; MEM, minimal essential medium; HA, hemagglutinin.
suggesting that cell type-specific differences in the function of the MEK-ERK signaling module might exist.

Despite these results, comparable studies in renal epithelial cells are not available to date. We have recently reported a differential regulation of ERK1, ERK2, and c-Jun NH2-terminal kinase 1 (JNK1) activity in dedifferentiated MDCK-C7/Focus (MDCK-C7F) cells as compared with their parental epithelial MDCK-C7 cells (22). Cloned MDCK-C7 cells, when grown for 2 weeks in alkaline (pH 7.7) culture medium, dedifferentiate, exhibit a spindle-shaped morphology with long dendrite-like protrusions, and lack contact inhibition as well as monolayer formation (22, 23). When subsequently cultured in standard medium (pH 7.4) MDCK-C7F cells maintained their altered phenotype (22, 23). Utilizing this renal epithelial cell model, we obtained a substantially increased ERK2 activity in quiescent and serum-treated dedifferentiated MDCK-C7F cells as compared with their parental epithelial MDCK-C7 cells (22). In contrast, JNK1 activity, which represents one member of the stress-activated protein kinase family of MAPKs, was slightly but consistently decreased in both quiescent and anisomycin-stimulated MDCK-C7F cells (22). Furthermore, differential activation of ERK2 and JNK1 was accompanied by an inhibition of serum-induced MDCK-C7F cell proliferation (22). Thus, transient exposure of epithelial MDCK-C7 cells to alkaline stress leads to cell dedifferentiation and growth inhibition associated with increased basal and serum-stimulated ERK2 activation but decreased JNK1 activity.

Based on our findings in dedifferentiated MDCK-C7F cells as compared with their parental epithelial MDCK-C7 cells, we hypothesized a negative regulatory function of MEK1 in renal epithelial cell differentiation and proliferation. To verify this hypothesis we utilized a constitutively active MEK1 construct (18) and studied its effects on cell differentiation and proliferation in epithelial MDCK-C7 cells. Here we report that stable expression of a hemagglutinin epitope-tagged constitutively active MEK1 mutant (S218D/S222D mutant) in MDCK-C7 cells leads to epithelial dedifferentiation as well as to a reduction of cell proliferation. This report provides evidence for a MEK1-ERK2-induced epithelial to mesenchymal transition of MDCK-C7 cell differentiation, associated with an attenuation of cell proliferation.

EXPERIMENTAL PROCEDURES

Materials—Leupeptin and pepstatin A were purchased from Peptide Institute Inc. (Osaka, Japan). PD098059 from Calbiochem-Novabiochem (Nottingham, UK). Cell culture media were from Sigma, and [γ-32P]ATP (5500 Ci/mmol) from DuPont NEN (Vienna, Austria). Protein A-Sepharose was purchased from Pharmacia Biotech Inc., Hygromycin B from Boehringer Mannheim (Vienna, Austria). Lipofectin reagent was obtained from Life Technologies, Inc. The polyclonal antibody HA.11, raised against the hemagglutinin epitope CYPDYVPDYASL, was purchased from Babco (Richmond, CA). The monoclonal antibody against cytokeratin was obtained from Progen (Heidelberg, Germany), and all other reagents were obtained from Sigma.

Cell Culture—Experiments were carried out using cloned wild-type MDCK-C7 cells or their stably transfected counterparts grown on plastic Petri dishes in minimal essential medium (MEM) with Earl’s salts, nonessential amino acids, and l-glutamine at a pH of 7.4 (22, 23). MEM was supplemented with 10% fetal calf serum (FCS), 26 mM NaHCO3, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37 °C in 5% CO2, 95% air, humidified atmosphere, and split in a ratio 1:10, twice a week (untreated and mock-transfected MDCK-C7 cells) or 1:6, once a week (MDCK-C7 cells stably transfected with constitutively active MEK1). After growth to a subconfluent state, cells were washed once, made quiescent by 24 h of incubation in FCS-free medium, and were then used for experiments.

Stable Transfection—MDCK-C7 cells (106 cells/10-cm plate) were cotransfected by the Lipofectin technique with 2.5 μg of pREP4 expression vector (Invitrogen) together with either 12.5 μg of the empty pCE expression vector (mock-transfected cells) or 12.5 μg of the pECE expression vector containing a hemagglutinin (HA) epitope-tagged constitutively active MEK1 mutant (18). The constitutively active mutant of MEK1 (CA-MEK1) was designed by substitution of the regulatory phosphorylation sites, Ser218 and Ser222, with aspartic acid (S218D/S222D mutant) as described previously (18). 48 h after transfection, cells were subjected to hygromycin B selection (50 μg/ml) to killed nontransfected cells. Stable clones were picked up for analysis and passaged once a week. For stimulation protocols, quiescent MDCK cell clones were transfected with either CA-MEK1 or the empty vectors were incubated in the presence of 10% FCS for the respective periods of time and compared with unstimulated controls.

Western Blot Analysis—Transfected MDCK-C7 cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μM pepstatin A, 1% Triton X-100) for 25 min at 4 °C. Insoluble material was removed by centrifugation at 12,000 × g for 15 min at 4 °C. For the examination of cytokeratin and vimentin protein expression only, cells were harvested into 1 × Laemmli buffer, and total cell homogenates were analyzed. The protein content was determined using a microbichoninic acid assay (Pierce) with BSA as the standard. Cell lysates were matched for protein, separated on 12% gel SDS-PAGE, and transferred to a polyvinylidene difluoride microporous membrane. Subsequently membranes were blotted with one of the following specific antibodies: anti-P-ERK1,2, which detects phosphorylated tyrosine 204 of both ERK1 and ERK2 (New England Biolabs, Beverly, MA), anti-MEK1 (generous gift of J. Pouyssegur, Nice), anti-HA.11 (Babco, Richmond, CA), anti-cytokeratin (Progen, Heidelberg, Germany), or anti-vimentin (Sigma). The primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (for ERK, MEK, and HA.11 antibodies) or horseradish peroxidase-conjugated goat anti-mouse IgG (for cytokeratin and vimentin antibodies) visualized by Amersham Corp. ECL system after intensive washing of the sheets or, when anti-P-ERK1,2 antibodies were applied, using alkaline phosphatase-conjugated goat anti-rabbit IgG visualized by Photootope™ chemiluminescent Western detection system (New England Biolabs).

Immunoprecipitation—Transfected MDCK-C7 cells were washed three times with ice-cold PBS and lysed in ice-cold Triton X-100 lysis buffer for 25 min at 4 °C. Insoluble material was removed by centrifugation at 12,000 × g for 15 min at 4 °C. The protein content was determined using a microbichoninic acid assay (Pierce) with BSA as the standard. Cell lysates were matched for protein and precleared with 2 μl of preimmune serum preadsorbed to 50 μl of protein A-Sepharose, coating for 1 h at 4 °C. The precleared supernatants were further incubated overnight either with 2 μl of a polyclonal antibody recognizing only ERK2 (generous gift of J. M. Dunn, Milwaukee) (24, 25) or with 4 μl of a polyclonal antibody recognizing p45 MEK1 (generous gift of J. Pouyssegur, Nice) (13) preadsorbed to protein A-Sepharose. Immunocomplexes were then used to measure ERK2 or MEK1 activity.

ERK2 Activity Assay—For measurement of ERK2 activity the respec-
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**Fig. 3.** The synthetic MEK inhibitor PD098059 inhibits basal and serum-stimulated ERK phosphorylation in mock- and CA-MEK1-transfected MDCK-C7 cells. Subconfluent MDCK-C7 cells transfected either with empty vectors (Mock) or with CA-MEK1, clone 1 (CA-MEK1/1) were made quiescent for 24 h as described under "Experimental Procedures." Thereafter cells were stimulated for 10 min with 10% FCS alone, 100 μM of the MEK inhibitor PD098059 alone (30 min + 10 min), or in the presence of both 100 μM PD098059 (30 min prior to preincubation) and 10% FCS (10 min). Phosphorylation of ERKs was determined by Western blot analysis as described under "Experimental Procedures" utilizing an anti-phos-ERTK antibody, which detects phosphorylated tyrosine 204 of both ERK1 and ERK2. Unphosphorylated ERK2 protein served as a negative control; phosphorylated ERK2 protein was utilized as a positive control (data not shown). Asterisks indicate the phosphorylated ERK isoforms (ERK1* and ERK2*, respectively). One representative Western blot of three separate experiments is depicted.

**Fig. 2.** ERK1,2 phosphorylation and ERK2 and MEK1 activation in CA-MEK1-transfected MDCK-C7 cells. A, six hygromycin B-selected, CA-MEK1-transfected cell clones (CA-MEK1/1 to 1/6) and one out of four hygromycin B-selected, mock-transfected control clones were grown in the presence of 10% FCS, analyzed by Western blotting, and probed with an anti-phospho-ERK antibody, which detects phosphorylated tyrosine 204 of both ERK1 and ERK2, as described under "Experimental Procedures." Unphosphorylated ERK2 protein served as a negative control, and phosphorylated ERK2 protein was utilized as a positive control (data not shown). Asterisks indicate the phosphorylated ERK isoforms (ERK1* and ERK2*, respectively). One representative Western blot of three separate experiments is depicted. B, subconfluent mock-transfected MDCK-C7 cells (Mock) and cell clone 1 of CA-MEK1-transfected MDCK-C7 cells (CA-MEK1/1) were made quiescent for 24 h (Ø FCS) or grown for 24 h in the presence of either 0.5% FCS or 10% FCS. ERK2 activity was determined in these cell lysates by immunoprecipitation of ERK2 and measuring its ability to phosphorylate MBP as a substrate. In lane 1 the experiment was performed in the absence of cell lysate (Ø Lysate). The results from one representative ERK2 activity assay of four separate experiments are depicted. C, MEK1 activity was determined by its ability to activate recombinant ERK2 (generous gift of D. Templeton, Cleveland) and hence phosphorylate MBP. The immunocomplexes were first incubated in kinase buffer containing 10 μg/ml recombinant ERK2 and 50 μM ATP at 30 °C for 30 min. Thereafter, MBP was added together with 10 μCi of [γ-32P]ATP and incubated for another 15 min at 30 °C. The reaction was terminated by addition of an equal volume of 2 × Laemmli buffer. Samples were boiled for 3 min and subjected to 12% gel SDS-PAGE. The gels were stained in Coomassie Brilliant Blue, dried, and exposed for 1–2 h to Amersham Hyperfilm MP at −70 °C with intensifying screens. In addition, kinase activity was determined by cutting the MBP bands and measuring the radioactivity in a liquid scintillation counter.

**MEK1 Activity Assay—**MEK1 activity was determined by its ability to activate recombinant ERK2 (generous gift of D. Templeton, Cleveland) and hence phosphorylate MBP. The immunocomplexes were first incubated in kinase buffer containing 10 μg/ml recombinant ERK2 and 50 μM ATP at 30 °C for 30 min. Thereafter, MBP was added together with 10 μCi of [γ-32P]ATP and incubated for another 15 min at 30 °C. The reaction was terminated by addition of an equal volume of 2 × Laemmli buffer. Samples were boiled for 3 min at 90 °C and subjected to 12% gel SDS-PAGE. The gels were stained with Coomassie Brilliant Blue, fixed, and dried for 3 h at 70 °C. Finally the dried gels were exposed for 1–2 h to Amersham Hyperfilm MP at −70 °C with intensifying screens. Furthermore, kinase activity was determined by cutting the MBP bands and measuring the radioactivity in a liquid scintillation counter.

**Immunofluorescence Microscopy—**Untransfected, mock-transfected, and CA-MEK1-transfected MDCK-C7 cells were plated onto Flexiperm-slides (Heraeus, Hanau, Germany) and were rinsed twice in PBS before being fixed for 15 min at room temperature in a freshly prepared solution of 4% paraformaldehyde, PBS. Thereafter, slides were rinsed twice in PBS, and cells were permeabilized by exposure to 0.5% Triton X-100 and stored in PBS containing 0.5% Triton X-100. The cell monolayer was permeabilized by exposure to 0.5% Triton X-100. The permeabilized monolayers were incubated with primary antibodies for 2 h and then with FITC-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 45 min.

**Fig. 4.** MEK1-induced Dedifferentiation of Epithelial MDCK-C7 Cells.
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RESULTS AND DISCUSSION

Stable Transfection of MDCK-C7 Cells with CA-MEK1 Is Associated with Increased ERK1/2 Phosphorylation and Increased ERK2 Activity—When grown for 2 weeks in alkaline (pH 7.7) culture medium, cloned MDCK-C7 cells dedifferentiate, exhibit spindle-shaped morphology, and lack contact inhibition as well as monolayer formation (22, 23). They maintain their altered phenotype when subsequently cultured in standard medium (pH 7.4) (22, 23). With respect to the activation of different members of the MAPK family of protein kinases, we have recently reported that these dedifferentiated MDCK-C7Focus (MDCK-C7F) cells show increased basal and serum-stimulated ERK2 stimulation but slightly decreased basal and anisomycin-induced JNK1 activity (22). To evaluate the role of the MEK1-ERK2 signaling module in epithelial differentiation/dedifferentiation of MDCK-C7 cells, we performed stable transfections of MDCK-C7 cells utilizing a HA epitope-tagged constitutively active MEK1 mutant (S218D/S222D mutant) (18), polyonal MDCK-C7 cells with typical epithelial growth characteristics and ultimately monolayer formation. B, pleiomorphic alkali-dedifferentiated MDCK-C7F cells, exhibiting spindle-shaped morphology, lack of epithelial monolayer formation, and poor adhesion to the culture support. C, mock-transfected MDCK-C7 cells with typical epithelial growth characteristics similar to cloned wild-type MDCK-C7 cells. D, stable expression of HA-tagged CA-MEK1, clone 1 (CA-MEK1/1) induces cell dedifferentiation comparable to the one obtained in MDCK-C7F cells after transient alkaline stress (as depicted in B). Bar = 200μm.

FIG. 4. Morphology of epithelial MDCK-C7 cells, alkali-dedifferentiated MDCK-C7F cells, and mock-transfected and CA-MEK1-transfected MDCK-C7 cells. Phase-contrast micrograph of subconfluent MDCK-C7 cells (A), MDCK-C7F cells (B), mock-transfected (C), and CA-MEK1-transfected (D) MDCK-C7 cells. A, flat, polygonal MDCK-C7 cells with typical epithelial growth characteristics and ultimately monolayer formation. B, pleiomorphic alkali-dedifferentiated MDCK-C7F cells, exhibiting spindle-shaped morphology, lack of epithelial monolayer formation, and poor adhesion to the culture support. C, mock-transfected MDCK-C7 cells with typical epithelial growth characteristics similar to cloned wild-type MDCK-C7 cells. D, stable expression of HA-tagged CA-MEK1, clone 1 (CA-MEK1/1) induces cell dedifferentiation comparable to the one obtained in MDCK-C7F cells after transient alkaline stress (as depicted in B). Bar = 200μm.
which is recognized by the polyclonal rabbit antibody HA.11. In addition, a polyclonal antibody raised against the MEK1 Xenopus NH2-terminal peptide was used to compare the level of expression of the ectopically expressed kinase with that of the endogenous one. Fig. 1 (left) shows that the polyclonal HA.11 antibody recognizes a 47-kDa protein only in MDCK-C7 cells transfected with constitutively active MEK1 (CA-MEK1/clone 1). In contrast, the polyclonal MEK1 antibody recognizes a single protein of 45 kDa in cells transfected with the empty vectors (mock-transfected cells) and a doublet of 45 and 47 kDa in transfected MDCK-C7 cells (Fig. 1, right). Altogether we obtained three MDCK-C7 clones (clones 1, 4, and 5) carrying the HA-tagged constitutively active MEK1 construct (CA-MEK1/1, 1/4, 1/5) out of a total of six hygromycin B-selected clones (CA-MEK1/1 to 6). Utilizing a polyclonal phosphospecific ERK antibody, which detects phosphorylated tyrosine 204 of both ERK1 and ERK2, we found that especially ERK2 phosphorylation was substantially augmented in the three CA-MEK1-positive MDCK-C7 cell clones when compared with mock-transfected cells (Fig. 2A). In addition, and in contrast to quiescent mock-transfected MDCK-C7 cells, ERK2 activity was increased 5.5-fold in quiescent MDCK-C7 cells stably transfected with HA-tagged CA-MEK1 (Fig. 2B). When grown for 24 h in the presence of 0.5% or 10% FCS, ERK2 activity was stimulated 2.4- and 1.5-fold in MDCK-C7 cells expressing the CA-MEK1 construct as compared with mock-transfected cells (Fig. 2B). Thus, stable transfection of MDCK-C7 cells with a constitutively active MEK1 mutant results in an increased phosphorylation of ERK1 and ERK2, which coincided with increased basal and serum-stimulated enzymatic ERK2 activity. Measurements of MEK1 activity by determining the ability of immunoprecipitated MEK1 to activate recombinant ERK2 and hence phosphorylate MBP confirmed these results. As depicted in Fig. 2C, MEK1 activity was 3.0-fold higher in quiescent CA-MEK1-transfected cells as compared with their mock-transfected counterparts.

Constitutively Active MEK1 Is Necessary for Increased ERK1 and ERK2 Phosphorylation in Stably Transfected MDCK-C7 Cells—PD098059, a synthetic inhibitor of the mitogen-activated protein kinase cascade, has been reported to selectively inhibit the ERK activator MEK, without significant inhibitory activity on ERK itself (26). Inhibition of MEK by PD098059 prevented activation of ERK and subsequent phosphorylation of ERK substrates both in vitro and in intact Swiss 3T3 fibroblasts (26). In addition, PD098059 completely blocked nerve growth factor-induced neurite formation in PC-12 cells without altering cell viability (27). As shown in Fig. 3, 100 μM PD098059 almost completely abolished basal ERK1 and ERK2 phosphorylation in mock-transfected MDCK-C7 cells as well as in cells stably transfected with constitutively active MEK1. Furthermore, when both cells, mock-transfected as well as CA-MEK1/1 cells, were preincubated with 100 μM PD098059 for 30 min prior to the addition of 10% FCS for 10 min, PD098059 strongly inhibited serum-induced ERK1 and ERK2 phosphorylation (Fig. 3), suggesting that active MEK1 is necessary for increased ERK1 and ERK2 phosphorylation.

Stable Transfection of MDCK-C7 Cells with a Constitutively

clonal anti-cytokeratin antibody. Subconfluent cells were grown in the presence of 10% FCS, fixed, rinsed thoroughly, and finally stained with a monoclonal anti-cytokeratin antibody recognizing type II cytokeratins 5 and 8, as described under “Experimental Procedures.” A and B, MDCK-C7 cells transfected with empty vectors. C and D, MDCK-C7 cells transfected with constitutively active MEK1 (clone 1). Photographs were taken using a Zeiss 20× objective. Representative immunofluorescence pictures from one out of four independent experiments are shown.

Fig. 5. Indirect immunofluorescence staining of mock-transfected and CA-MEK1-transfected MDCK-C7 cells with a mono-
Active MEK1 Mutant Is Associated with Epithelial Dedifferentiation—Transient alkaline stress leads to differential activation of mitogen-activated protein kinases associated with alterations in epithelial cell morphology (22). To determine whether or not the MEK-ERK module could be directly involved in the regulation of epithelial MDCK-C7 cell differentiation, we first investigated the morphology of mock-transfected cells and compared them with the stable cell clone MDCK-C7 CA-MEK1/1 bearing the constitutively active MEK1 mutant. Both untransfected (Fig. 4A) as well as mock-transfected MDCK-C7 cells (Fig. 4C) showed a typical epithelial morphology growing in islands of flat, polygonal cells, which ultimately form confluent monolayers and are capable of dome formation due to a vectorial transport of salt and water from the apical to the basolateral side at the base of the culture dish. This typical epithelial phenotype was also observed in cell clones 1/2, 1/3, and 1/6, which do not express constitutively active MEK1 and hence do not show increased ERK1 and ERK2 phosphorylation (see Fig. 2A). In contrast, only MDCK-C7 cell clones successfully transfected with CA-MEK1, namely clones CA-MEK1/1 (depicted in Fig. 4D), CA-MEK1/4 and CA-MEK1/5, were pleomorphic, exhibited a spindle-shaped morphology, never formed an organized epithelial monolayer, and thus resembled alkali-differentiated MDCK-C7Focus cells (Fig. 4B) (22). Therefore, expression of a constitutively active MEK1 mutant, which increases basal and serum-stimulated ERK2 activity, leads to a dramatic change in MDCK-C7 cell phenotype consistent with epithelial dedifferentiation.

Epithelial cells are usually cuboidal in shape, but may also be columnar or squamous. The tissue phenotype is defined by a high degree of apical-basal polarity. Mesenchymal cells, on the other hand, are elongated or stellate in shape and are characterized by their expression of vimentin intermediate filaments (28). Epithelia produce intermediate filaments of the cytokeratin type with a few exceptions, such as the cultured renal MDCK cell line, that contains both vimentin and keratin (29, 30). As mesenchymal cells would be expected to express only vimentin intermediate filaments, we next studied the expression of cytokeratin and vimentin in untransfected and mock-transfected MDCK-C7 cells as compared with MDCK-C7 cells stably transfected with CA-MEK1 using immunofluorescence and Western blot techniques. Indirect immunofluorescence staining revealed a loss of cytokeratin expression (Fig. 5) but an increase in vimentin expression (Fig. 6) in dedifferentiated MDCK-C7 cells bearing CA-MEK1 as compared with mock-transfected cells. While in mock-transfected MDCK-C7 cells vimentin intermediate filaments were typically surrounding the nucleus and extending from this perinuclear region toward the periphery (Fig. 6B), CA-MEK1-transfected cells showed a strong and homogeneous staining throughout the cytoplasm (Fig. 6D). These findings were confirmed by Western blot analysis (Fig. 7). SDS-PAGE of subconfluent, protein-matched cells and subsequent blotting with either a monoclonal cytokeratin antibody or a monoclonal vimentin antibody again showed lack of cytokeratin expression (Fig. 7A) but increased vimentin expression (Fig. 7B) in MDCK-C7 cells transfected with CA-MEK1. Moreover, only successfully transfected cell clones CA-MEK1/1, 1/4, and 1/5 lack cytokeratin expression, while clones...
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Inhibition of Cell Proliferation in MDCK-C7 Cells Expressing Constitutively Active MEK1—Transient alkaline stress and subsequent culture under neutral pH does not only lead to differential activation of mitogen-activated protein kinases associated with stable alterations of epithelial MDCK-C7 cell morphology but also to an inhibition of serum-induced cell proliferation (22). To determine whether the MEK-ERK module could be involved in the regulation of MDCK-C7 cell proliferation, we next investigated the growth characteristics of MDCK-C7 cells transfected with the empty vectors (mock-transfected cells) and compared them with the stable cell clone MDCK-C7 CA-MEK1/1. When mock-transfected MDCK-C7 cells were made quiescent for 24 h in the absence of serum and then stimulated with 10% FCS, cell numbers increased 4.6-fold after 2 days, 5.9-fold after 3 days, and 12.9-fold after 5 days of stimulation (Fig. 8). In contrast, when quiescent MDCK-C7 cells stably transfected with constitutively active MEK1 were stimulated with 10% FCS, cell numbers did not change within the first 2 days and increased only 1.2- and 4.6-fold after 3 and 5 days, respectively (Fig. 8), suggesting that increased MEK1 activity and increased stimulation of ERK2 are not only associated with dedifferentiation of MDCK-C7 cells but also with a delayed and reduced cell proliferation.

To confirm these data we finally performed flow cytometric DNA analysis. The experiments presented in Fig. 9 demonstrate that stable expression of constitutively active MEK1 significantly decreased the proportion of cells moving from G0/G1 to G2/M phase of the cell cycle. Single cell suspensions of untransfected, mock-transfected, and CA-MEK1/1-transfected MDCK-C7 cells grown in the presence of 10% FCS were prepared as described under “Experimental Procedures,” and the number of cells in G2/M phase of the cell cycle was determined by flow cytometric DNA analysis. All values given are arithmetic means ± S.E. of n = 4–6 experiments. No error bars are shown where symbols are larger than the S.E.

The representative FACS profiles depicted in Fig. 10 reveal that...
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CA-MEK1-transfected cells accumulate in G0/G1, hence leading to a decrease in G2/M cells. Stable transfection of MDCK-C7 cells with CA-MEK1 led to a significant increase in the fraction of cells present in G0/G1 (70.0 ± 2.9%) as compared with mock-transfected (49.5 ± 4.6%, p < 0.01) and untransfected cells (59.2 ± 2.0%, p > 0.05). In contrast, analysis of the Sub-G0/G1 peak showed no significant differences in between untransfected (8.6 ± 2.1%), mock-transfected (12.7 ± 0.3%), and CA-MEK1-transfected MDCK-C7 cells (11.1 ± 2.4%), suggesting that there exists no evidence for increased apoptosis in CA-MEK1/1 cells. Moreover, no significant differences were found for S phase cell fractions when CA-MEK1/1-transfected cells were compared with mock-transfected and untransfected cells, respectively (data not shown). Thus, CA-MEK1-associated dedifferentiation of epithelial MDCK-C7 cells is accompanied by a reduction of cell multiplication due to a decrease in the proportion of cells moving from G0/G1 to G2/M.

In summary, we recently described dramatic alterations in the activation of certain MAPK in alkali-dedifferentiated MDCK-C7F cells as compared with their parental epithelial MDCK-C7 cells (22). To find more direct evidence whether or not the highly conserved MEK1-ERK2 signaling module is involved in the regulation of epithelial differentiation in MDCK-C7 cells, we utilized a constitutively active MEK1 mutant as well as a stable transfection approach. In contrast to fibroblasts where constitutively active MEK1 led to cell transformation (18–20), and in contrast to PC12 cells in which this mutant induced neuronal differentiation (19), our present results provide evidence for a role of the MEK1-ERK2 signaling module as a negative regulator of epithelial differentiation in MDCK-C7 cells. Whether reduction of MDCK-C7 cell proliferation represents a direct result of CA-MEK1 transfection or is an indirect effect of MDCK-C7 cell dedifferentiation remains to be elucidated. However, it is tempting to speculate that, depending on the presence of specific substrates in certain cell types, different members of the MAPK family of protein kinases could exert distinct signaling functions leading to their cell-specific effects on cell differentiation and/or proliferation.

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