MEK1 and MEK2, Different Regulators of the G1/S Transition*

Siegfried Ussar‡ and Tilman Voss§

From the Boehringer Ingelheim Austria GmbH, Dr. Boehringer Gasse 5-11, A-1121 Vienna, Austria

The ERK cascade is activated by hormones, cytokines, and growth factors that result in either proliferation or growth arrest depending on the duration and intensity of the ERK activation. Here we provide evidence that the MEK1/ERK module preferentially provides proliferative signals, whereas the MEK2/ERK module induces growth arrest at the G1/S boundary. Depletion of either MEK subtype by RNA interference generated a unique phenotype. The MEK1 knock down led to p21\(^{\text{cip1}}\) induction and to the appearance of cells with a senescence-like phenotype. Permanent ablation of MEK1 resulted in growth arrest at the G1/S boundary. Depletion of either MEK1 or MEK2 might, therefore, represent a switch mechanism between induction or inhibition of cell proliferation.

In this study we investigated to what extent the loss of MEK1 and MEK2 contribute to the divergent effects of ERK signaling on either induction or inhibition of cell proliferation in several cell lines. Complete inhibition of MEK1/2 activity with PD184352 resulted in G1 arrest and induction of apoptosis (8). The selective ablation of either subtype by RNAi, however, generated completely different effects. Loss of MEK1 protein resulted in sustained ERK activation, resulting in p21\(^{\text{cip1}}\) up-regulation. This led to a gradual appearance of cells with a senescence-like phenotype and eventually to a reduced colony-forming potential. In contrast, MEK2 depletion and the double knock down led to centrosome over-amplification and, thus, to multipolar spindle formation. These data showed that MEK1 and MEK2 activities have a different impact on the intensity and duration of ERK signaling and, thus, the biological response. Stimulus-dependent specific activation of either MEK1 or MEK2 might, therefore, represent a switch mechanism between induction or inhibition of cell proliferation.

MATERIALS AND METHODS

Reagents and Cell Culture—HCT-116, HT-29 (both colon cancer cell lines), AspC-1 (pancreatic cancer), and HS-27 (human foreskin fibroblasts) were maintained in Dulbecco’s modified Eagle’s medium containing 4500 mg/liter glucose supplemented with 10% fetal calf serum and supplements (0.1 nm nonessential amino acids, 2 mM glutamine, 1 mM sodium pyruvate (all stocks were from BioWhittaker)). Antibodies were obtained from Cell Signaling (ERK, Phospho-ERK (pERK), MEK, RKS, ribosomal S6 kinase)
**MEK1 and MEK2 in Cell Proliferation**

pMEK, cyclins D1, D3, pBR Ser-780, pBSK Thr-358/Ser-363, BD Biosciences (MEK1, MEK2, cyclin B1-fluorescin isothiocyanate), Santa Cruz (cyclins A and E, Fra-1, p27\(^{kip1}\)), Sigma (α- and γ-tubulin), and Calbiochem (p21\(^{cip1}\), pNucleophosmin Thr-199 antibody was a kind gift of Dr. Spevak.

**RNA Interference**—Two different sequences for each MEK1 and MEK2 were selected for the generation of siRNA oligonucleotides based on published suggestions (23). BLAST searches were performed to verify the specificity of the following sequences: MEK1, AAAGCAACTCATGGTTCATGC; MEK2, AAGGGATCGAT was used for control transfections. 100 nM siRNA duplexes (Dharmacon Research) were transfected into cells using LipofectAMINE 2000 (Invitrogen) following the manufacturer's instructions. For the double knock down 100 nM each siRNA was used. Suppression of MEK1, MEK2, and p21\(^{kip1}\) was verified by Western blotting with MEK1, MEK2, and p21\(^{kip1}\)-specific antibodies.

**Vector Construction**—Short hairpin (sh) RNA-expressing constructs (shMEK1, shMEK2) were based on the pSilencer 3.1-H1 neo vector (Ambion) containing a H1 promoter and a neomycin resistance gene. Sixty-four-bp-long inserts specific for MEK1 and MEK2 were designed (Ambion) containing a H1 promoter and a neomycin resistance gene. Sixty-four-bp-long inserts specific for MEK1 and MEK2 were selected for the generation of siRNA oligonucleotides based on Dr. Spevak.

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**Short hairpin (sh) RNA-expressing plasmid** was linearized using Scal before transfection. HCT-116 cells were transfected using LipofectAMINE 2000 following the manufacturer's instructions. Twenty-four hours after transfection cells were replated onto 10-cm dishes with 80,000 cells/dish in complete medium supplemented with 10% fetal calf serum. HCT-116 cells were lysed as described above, and total protein was extracted with a clear reduction of total pMEK. The increased phosphorylated, as a consequence of the MEK1 knock down, as indicated by a different region of MEK1 and MEK2 (data not shown).

**Depleting MEK1 and MEK2**—HCT-116 cells were transfected with the indicated siRNAs. [\(^{35}S\)Thymidine (5 μCi, Amersham Biosciences) was added per well 20 h before measurement. Cells were transfected onto filter plates, dried, covered with 25 μl of scintillation mixture, and sealed. Thymidine incorporation was measured with a scintillation counter (1450 Microbeta, Wallac).

**Proliferation Assay**—HCT-116 cells were seeded onto 96-well plates with 2000 cells/well, cultured for 24 h, and transfected with the indicated siRNAs. After siRNAs had been incubated with 20 μl of fluorescein isothiocyanate-labeled cyclin B1 antibody (BD Biosciences) in 100 μl of PBS for 30 min at 4 °C. Cells were incubated with 0.25% Triton X-100/PBS for 5 min and PBS-washed, and DNA was stained with propidium iodide (10 μg/ml) supplemented with 1 mg/ml RNase for 20 min at room temperature in the dark. DNA content and cyclin B1 levels were determined using a FACSCalibur (BD Biosciences) with the CellQuest software package.

**Immunofluorescence**—HCT-116, HT-29, and Aspc-1 were cultured in 4 chamber slides (BD Biosciences) for 24 h, transfected as described, and maintained for further 48 h. Cells were washed with PBS and fixed with methanol/acetone (1:1) for 10 min at −20 °C. Slides were blocked with 10% goat serum in 1% bovine serum albumin, PBS. The primary antibodies used were: α-Tubulin (1:1000; Sigma), cyclin D1 (1:100; Cell Signaling), and cyclin B1 (1:100; BD Biosciences) were used according to the manufacturer's suggestions. A Texas Red (549 nm)-labeled secondary monoclonal anti-mouse antibody (Molecular Probes) was used. α-Tubulin staining was performed with a fluorescein isothiocyanate-labeled monoclonal α-tubulin antibody (1:75; Sigma) for 20 min. Cells were washed with 0.1% Triton X-100/PBS for 30 min, mounted in Mowiol (Calbiochem) containing 50–100 ng/ml 4,6-diamidino-2-phenylindole and examined under epifluorescent illumination. Photos were either taken with an Axiosplan2 or a LSM510 (confocal), both from Zeiss with an 63× objective.

**Metabolic Labeling of Cyclin D1—HCT-116** were transfected with the indicated siRNAs 48 h before labeling. Cells were washed twice with PBS and transfected to methionine/cysteine-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 24 μg/liter cysteine containing 200 μCi/ml [\(^{35}\)S]methionine (555 MBq/ml, Amersham Biosciences). Cells were labeled for 30 min, washed twice with PBS, and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HCT-116 cells were lysed as described above, and labeled cyclin D1 was precipitated with mouse anti-cyclin D1 antibody (1:100; Cell Signaling) overnight. The antibody was bound to protein G-beads (Sigma) for 2 h, washed 5 times with lysis buffer, and resolved on denaturing 10% Tris-glycine gels, transferred to Immobilon-P membranes, and visualized by autoradiography.

**RESULTS**

**MEK1 and MEK2 Act Differently on ERK Activation—Depletion of MEK1, MEK2, or both in HCT116 colon cancer cells using RNAi was very efficient as revealed by Western blot analysis (Fig. 1A). The minimal amount of siRNA required to get this down-regulation was determined in a titration experiment. The transfection procedure itself did not affect MEK1 and MEK2 levels (Fig. 1A, controls). Nevertheless, slightly reduced MEK1 protein was detected 48 h after transfection in MEK2-deficient (siMEK2) and control cells. Furthermore MEK2 protein levels increased during the time span observed in control and MEK1-deficient (siMEK1) cells. Forty-eight hours after transfection neither MEK1 nor MEK2 was detectable with subtype-specific antibodies. Slightly reduced knock down results were obtained with the alternative siRNAs, targeting a different region of MEK1 and MEK2 (data not shown).**

The effect of MEK depletion on MEK and ERK activity was assessed in Western blots using antibodies specifically recognizing phosphorylated and, thus, activated MEK1/2 and ERK1/2. Either knock down resulted in a total loss of the respective phospho-MEK (pMEK) levels (Fig. 1B). Within the first 48 h after transfection no additional MEK2 was phosphorylated, as a consequence of the MEK1 knock down, as indicated by a clear reduction of total pMEK. The increased
were performed with an antibody specific for Fra-1. Sustained ERK activation in siRNA-transfected cells, Western blots bands relative to GAPDH control is shown. GAPDH was used for the loading control. Quantification of the pERK times, and Western blots with anti-pERK antibody were performed. Samples were taken 48 h after transfection. The lines in A and B indicate rearranged parts of the same blot. A, knock down efficiency was assayed by Western blots using subtype-specific antibodies. The right panel includes further controls (no transfection (mock), and the control oligo (siControl)), to prove that the transfection itself did not affect MEK levels. Samples were taken 48 h after transfection. B, to assay MEK and ERK activation in siRNA-transfected cells Western blots with anti-pMEK and anti-pERK antibodies were performed. The 35-kDa band represents mitotically phosphorylated nucleophosmin cross-reacting with the pMEK antibody.

pERK levels were analyzed as a readout for MEK activity upon MEK1 or MEK2 knock down. In the control transfection pERK levels decreased over time as a consequence of increasing cell density and contact inhibition. This observation could be further confirmed since the amount of G1 cells continuously increased, as verified by fluorescence-activated cell sorter analysis (data not shown). pERK levels were rather low in the MEK1 knock down 24 h after transfection but exceeded the control levels at 48 and 72 h (Fig. 1B). This suggested an over-compensation by MEK2. In contrast, the MEK2 and, in particular, the double knock down resulted in clearly reduced pERK levels.

To investigate whether ablation of MEK1 and MEK2 also interferes with the activation kinetics of ERK, we performed re-stimulation experiments that were carried out 48 h after transfection. The control cells exhibited the expected biphasic behavior (10) with pERK peak maxima at 5 and 20 min followed by a decrease at later times (Fig. 1C). The MEK2 knock down showed only one maximum around 10–20 min followed by a slow decrease. The MEK1 knock down, in contrast, resulted in constitutively high ERK activation from the beginning throughout the entire observation period. Since the sustained activation of ERK would cause a change in the expression of downstream targets, we decided to investigate the levels of the AP-1 family member Fra-1. Fra-1 expression was shown to require sustained ERK activity (14) in a c-Fos-dependent process. Indeed we could observe an induction of Fra-1 (Fig. 1D) beginning 24 h after transfection. This sustained strong ERK activity, therefore, might be attributed to the enforced activation of ERK by MEK2.

These findings were further supported by previous reports that showed MEK2 to be an ~7-fold better activator of ERK than MEK1 (25). This would explain the above-described sustained activation of ERK by MEK2 in MEK1 knock down cells.

Colony-forming assays with HCT-116 cells transfected with the respective shRNA-expressing plasmids demonstrated an important role of MEK1 in long term cell proliferation, as recently published (22). Cells transfected with the pSilencer vector expressing a short hairpin targeting MEK1 (shMEK1) formed significantly fewer colonies (Fig. 2A). Furthermore, none of 24 single cell clones derived from this transfection showed reduced levels of MEK1, indicating that in these cells only the neomycin resistance gene had integrated. In contrast, at least six of 24 clones with clearly reduced MEK2 levels were obtained from the transfection with the respective shMEK2 plasmid (selected clones shown in Fig. 2B).

To address the early effects of MEK depletion on cell proliferation, thymidine incorporation was examined in transiently transfected cells. A significant reduction of the proliferation rate was observed in both the MEK2 and the double knock down cells (Fig. 3A). In contrast to the colony-forming assay, no decreased proliferation was observed in MEK1-deficient cells within 72 h after transfection. The specific MEK inhibitor PD184352 caused a complete proliferation arrest and even a loss of cells (Fig. 3A).

Cell cycle analyses were performed 48 h after transfection to further characterize the observed proliferation differences. A
slight but significant increase in the S-phase population was observed in the MEK1 knock down (Fig. 3B), whereas the MEK2-deficient cells exhibited an obvious increase in G2/M. This key effect of G2/M delay upon MEK2 depletion was verified using an alternative siRNA (Fig. 3, B and C, siMEK2_2). The double knock down resembled the MEK2 knock down, demonstrating the dominant effect of MEK2 loss. A detailed analysis by cyclin B1/DNA double-staining (Fig. 3C) revealed that the MEK2-deficient cells had a nearly 2-fold increase in the tetraploid cell population and, within this population, a delay at a late mitotic stage. In contrast, the addition of 10 μM PD184352 for 24 h resulted in a clear G1 arrest (Fig. 3B). One representative experiment of four is shown.

MEK Depletion Alters Cyclin D Stability and Transcription—The G1 arrest induced by PD184352 is due to the inhibition of cyclin D transcription (8). We, therefore, analyzed the effect of MEK1 and MEK2 depletion on cyclin D1 and D3 levels between 24 and 72 h after transfection (Fig. 4A). In control cells cyclin D1 and D3 levels decreased over time, following the decreasing pERK levels (Fig. 1B). Unexpectedly, upon depletion of either MEK1 or MEK2, cyclins D1 and D3 were elevated (Fig. 4A; cyclin D2 was not detectable in HCT-116 cells; data not shown). The cyclin D kinetics were clearly different for the MEK1 and the MEK2 knock down. MEK1 deficiency induced a gradual accumulation of cyclin D1 levels, reaching a maximum 72 h after transfection. In contrast, the MEK2-deficient cells exhibited an immediate induction of high cyclin D1 levels. The double knock down seemed to integrate both effects as the cyclin D1 levels were slightly higher at 24 h compared with the MEK1 knock down but further increased over time. Induction of cyclin D1 upon MEK1 and MEK2 depletion was observed in HT-29, AspC1, and HS-27 cells too (data not shown). Although the double knock down had reduced cyclin D3 compared with the single knock downs, cyclin D3 levels neverthe-


less showed basically the same kinetics as cyclin D1. Therefore, in all following experiments only cyclin D1 levels were analyzed. These observations were identical when alternative siRNA oligos were used, proving that they are not the result of RNAi-mediated off-target effects (data not shown).

The gradual accumulation of cyclin D1 in MEK1-deficient cells and the consistently high levels in MEK2-deficient cells indicated possible changes in either cyclin D1 protein stability or transcription. Pulse-chase experiments performed 48 h after transfection demonstrated a clear stabilization of cyclin D1 in the MEK1 and the double knock down (t1/2 = ~40 min versus ~20 min in the control and MEK2 knock down, Fig. 4B). Similar stabilization kinetics of cyclin D had been shown earlier for the inactivation of GSK3β (26). The cyclin D1 signal was nearly absent after pretreatment of cells with 10 μM PD184352 for 24 h, as described earlier (8).

GSK3β is known to be regulated by Akt and to control the degradation of cyclin D. Therefore, we investigated the total amount and phosphorylation status of these two regulators of cyclin D (Fig. 4C). Both the total amount and phosphorylation of GSK3β were increased in MEK1-deficient and double knock down cells from 48 h after transfection. However, MEK2 knock down also resulted in an increase of pGSK3β and total GSK3β compared with the control (Fig. 4C). Akt phosphorylation on Ser-473 remained unchanged in MEK1-deficient cells, whereas it was slightly decreased in MEK2 and the double knock down. However, pAkt Thr-308 was clearly increased in the MEK1 knock down.

Cyclin D1 protein was increased in MEK2-deficient cells at all time points, but the stability seemed to remain unchanged. Thus, the expression levels of cyclin D1 were determined using real time PCR (Fig. 4D). The transcription of cyclin D1 was highly increased in MEK2-deficient (>15-fold) and double knock down cells (10-fold), but even the MEK1 deficient cells had increased cyclin D1 mRNA levels (3-fold). The high expression of cyclin D1 in MEK2-deficient cells resulted in a high de novo synthesis of cyclin D1 protein during the chase period of the pulse-chase experiment (Fig. 4B), which could mimic a decreased stability. This, however, seems unlikely, because the half-life is in the same range as the one observed for the control transfection exhibiting the lowest transcription level (Fig. 4D).

The potentially different impact of MEK1 or MEK2 deficiency on either stabilization or transcription could explain the different cyclin D1 kinetics. MEK1 deficiency-induced stabilization led to a gradual accumulation of the protein, whereas the increased transcription in MEK2-deficient cells immediately led to increased protein levels. Moreover, the combination of stabilization and increased transcription in the double knock down could explain the cyclin D kinetics.

These data suggest that MEK1 and MEK2 have different and non-overlapping influences, resulting in increased cyclin D levels, as manifested in the double knock down featuring both increased transcription and protein stability. However, the observed stabilization of cyclin D could not be attributed to an obvious induction of GSK3β or Akt phosphorylation. Moreover, the increased levels of GSK3β itself indicated an additional regulatory system.

Increased CDK4/6 Activity Does Not Affect CDK2 Activity—The increased cyclin D1 levels suggested an increase in CDK4/6 activity. Phosphorylation of Rb at a CDK4/6-specific site (Ser-780) was used as a surrogate marker for CDK4/6 activity. Depletion of MEK1 and, in particular, MEK2 resulted in a qualitative and quantitative change of the regular Rb phosphorylation pattern compared with the control transfection (Fig. 5A). The most intense phosphorylation could be observed in the MEK2 knock down cells within the first 48 h after
transfection. The complex band pattern in all knock downs suggested additional phosphorylation on other sites. However, knock down of both proteins had only a moderate effect on CDK4/6 activity.

Cyclin E and cyclin A were analyzed to test for the efficient release of E2F upon Rb phosphorylation (Fig. 5A). Moderately increased levels of cyclin E were observed in all knock downs. A significant increase of cyclin A, particularly 48 h after transfection, was seen only in the MEK2 and the double knock down. To further investigate this we analyzed the phosphorylation of nucleophosmin at Thr-199, which has been shown to be a CDK2 target site (27). This phosphorylation differs from the one observed as cross-reactivity with the pMEK antibody. Phosphorylation of Thr-199 is required for the dissociation of nucleophosmin from the centrosomes and the induction of centrosome duplication during S phase, whereas the pMEK antibody recognizes mitotically phosphorylated nucleophosmin. Phosphorylation of Thr-199 was greatly increased in siMEK2 cells and the main ERK response gene to inhibit proliferation (30). Therefore, a MEK1/p21cip1 double knock down approach was used to probe for the role of p21cip1 in this context. The knock down efficiency was verified by Western blot using MEK1- and p21cip1-specific antibodies (data not shown). As expected the p21cip1 knock down resulted in increased CDK2 activity, but interestingly, phosphorylation of the CDK2 substrate nucleophosmin was not increased (Fig. 6C). The senescence-like phenotype was not seen in either the p21cip1 or the p21cip1/MEK1 double knock down. The p21cip1 induction upon MEK1 down-regulation was a key response. Thus, to confirm that this up-regulation was due to MEK1 deletion and not due to RNAi-mediated off-target effects, it was confirmed with the second MEK1 oligo (Fig. 6B). The loss of MEK1 activity, which is overcompensated by the forced activation of MEK2, resulted in sustained ERK activation and, thus, led to a gradual p21cip1-dependent induction of a senescence-like phenotype.

**MEK1 and MEK2 in Cell Proliferation**

**Fig. 3. MEK2 deficiency leads to reduced proliferation and a delay in G2/M.** A, proliferation assay (thymidine incorporation) of HCT-116 cells transfected with the indicated siRNAs. [3H]Thymidine was added 20 h before the indicated time points. Time points indicate hours after transfection. Each time point was prepared in triplicate. The curves shown are representative of three independent experiments. B, fluorescence-activated cell sorter analysis of DNA content performed 48 h after transfection with the indicated siRNAs. MEK2_1 and MEK2_2 represent the data for the two alternative MEK2 siRNAs. Cells treated with 10 μM PD184352 served as a control. C, Western blot analysis demonstrating the MEK2 knock down efficiency of the alternative MEK2 siRNA 48 h after transfection. D, propidium iodide and cyclin B1 fluorescence-activated cell sorter analysis 48 h after transfection. The tetraploid cyclin B negative cell population in MEK2-deficient cells is marked by a circle. The numbers indicate the relative amounts of the tetraploid cyclin B positive (upper right) and negative (lower right) cell populations that are increased in the MEK2 knock down cells.

Together these data showed that the MEK1 knock down resulted in continuously increased p21cip1 levels. The MEK2 knock down, in contrast, led to hyperactivated CDK4/6 and to an obviously CDK2-independent increase in nucleophosmin phosphorylation.

**p21kip1 Induction Is Responsible for a Senescence-like Phenotype of MEK1-deficient Cells—**MEK1-deficient cells contained a striking subpopulation of very large p21kip1-positive cells, reminiscent of senescent-like cells (6 cells per field versus 1–2 cells in control transfections, 10 fields counted at 10× magnification; Fig. 6A). The number of these large cells seemed to increase slightly between 24 and 72 h after transfection. No such cells were observed under any other knock down condition. p21kip1 is an important factor in cells undergoing senescence and the main ERK response gene to inhibit proliferation (30). Therefore, a MEK1/p21kip1 double knock down approach was used to probe for the role of p21kip1 in this context. The knock down efficiency was verified by Western blot using MEK1- and p21kip1-specific antibodies (data not shown). As expected the p21kip1 knock down resulted in increased CDK2 activity, but interestingly, phosphorylation of the CDK2 substrate nucleophosmin was not increased (Fig. 6C). The senescence-like phenotype was not seen in either the p21kip1 or the p21kip1/MEK1 double knock down. The p21kip1 induction upon MEK1 down-regulation was a key response. Thus, to confirm that this up-regulation was due to MEK1 deletion and not due to RNAi-mediated off-target effects, it was confirmed with the second MEK1 oligo (Fig. 6B). The loss of MEK1 activity, which is overcompensated by the forced activation of MEK2, resulted in sustained ERK activation and, thus, led to a gradual p21kip1-dependent induction of a senescence-like phenotype.

**MEK2 Deficiency Leads to Centrosome Abnormalities, Independent of CDK2 Activity—**The MEK2-deficient and the double knock down cells were shown to have increased phosphorylation of nucleophosmin at Thr-199 (Fig. 5A). This phosphoryla-
tion is a prerequisite for centrosome duplication (27). Because the MEK2 knockdown cells had been shown to exhibit an increased G2/M population (Fig. 3B), we wanted to investigate the impact of increased Thr-199 phosphorylation on cell cycle progression. Immunofluorescence studies (Fig. 7A) demonstrated a doubling of the mitotic index (8.7% versus 4.5%, 100 mitotic cells counted) in MEK2-deficient cells. Additionally, one-fourth of the mitotic cells had either tri- or tetrapolar spindles, whereas none of these spindles could be observed in the MEK1-deficient or in control cells (Fig. 7A, arrows). No other spindle abnormalities were observed. The double knockdown revealed the same features as the MEK2 knockdown. Because these cells were almost all cyclin B1-negative, this cell population most likely represents the cyclin B1-negative cells seen in the fluorescence-activated cell sorter profiles (Fig. 3C).

Confocal microscopy of cells stained with the centrosome marker γ-tubulin 48 h after transfection revealed an increased number of centrosomes in interphase (Fig. 7B, upper panel) and mitotic (Fig. 7B, lower panel) cells. This centrosome overduplication seemed to be responsible for the observed spindle abnormalities. These spindle abnormalities could also be observed in HT-29 and AspC-1 (data not shown), generalizing this observation. The second MEK2 siRNA oligo also induced these spindle abnormalities, but the number of abnormal spindles was reduced due to its reduced MEK2 knockdown efficiency (data not shown). These data indicated that the increased phosphorylation of nucleophosmin was accompanied by an over-duplication of centrosomes during S phase in several cell types. Furthermore, the increased phosphorylation of nucleophosmin at Thr-199 seemed to be independent of CDK2 activity, as shown in the p21cip1 and the p21cip1/MEK1 knockdown (Fig. 6C). The p21cip1/MEK1 knockdown was insufficient to induce these spindle abnormalities, supporting the interpretation that CDK4/6 is the driving force behind this phenotype.

The population of cells with aberrant spindles could not fully account for the doubled mitotic index of the MEK2-deficient cells. The MEK2 deficiency, therefore, had to also affect mitotic MEK/ERK activity. RSK-1 phosphorylation at the ERK target sites Thr-359 and Ser-363 was analyzed as readout for mitotic ERK activity. RSK-1 phosphorylation was significantly reduced in the MEK2-deficient cells (Fig. 7C). The p21cip1/MEK1 knockdown was insufficient to induce these spindle abnormalities, supporting the interpretation that CDK4/6 is the driving force behind this phenotype.

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DECREASE IN ERK PHOSPHORYLATION DIFFERED CLEARLY FROM THE G1 DOWN OF BOTH MEK1 AND MEK2 ACCOMPANIED BY A MASSIVE CELL CYCLE PROGRESSION IS PERTURBED. EVEN THE COMBINED KNOCKDOWN OF p21cip1, THE ALTERNATIVE MEK1 siRNA, OR lysates, 48 h AFTER TRANSFECTION WITH EITHER siRNAs, WERE STAINED 48 h AFTER TRANSFECTION WITH SPECIFIC ANTIBODIES FOR p21cip1 AND α-tubulin. DNA WAS VISUALIZED WITH 4,6-DIAMIDINO-2-PHENYLINDOLE (DAPI). THE BARS INDICATE 10 μM. B, CELL LYSATES, 48 h AFTER TRANSFECTION WITH EITHER p21cip1, THE ALTERNATIVE MEK1 siRNA, OR bOTH siRNAs WERE IMMUNOBLOTTED WITH ANTIBODIES SPECIFIC FOR THE RESPECTIVE PROTEIN TO VERIFY THE KNOCK DOWN EFFICIENCY. C, CELL LYSATES AS DESCRIBED IN B WERE USED FOR KINASE ASSAYS OF IMMUNOPRECIPITATED CDK2 USING HISTONE H1 AS A SUBSTRATE (LEFT PANEL). ALIQUOTS OF THESE LYSATES WERE ANALYZED BY WESTERN BLOTS USING AN ANTIBODY SPECIFIC FOR THR-199-PHOSPHO-NUCLEOPHOSMIN (RIGHT PANEL).

DISCUSSION

Extensive studies, reviewed by Roovers and Assoian (4), show that the mitogen-induced amplitude and duration of ERK activity regulates transcription of p21cip1 and cyclin D and thereby balances growth arrest and proliferation. Here we demonstrate that an imbalanced MEK1/MEK2 signaling as a consequence of RNAi-mediated knock down of either subtype leads to changes in the amplitude and duration of ERK activity. As a consequence, cyclin D and p21cip1 levels are changed, and cell cycle progression is perturbed. Even the combined knock down of both MEK1 and MEK2 accompanied by a massive decrease in ERK phosphorylation differed clearly from the G1 arrest induced by a specific inhibitor like PD184352 via the transcriptional inhibition of cyclin D (8). We believe that these rather unexpected differences are likely to result from the fact that the MEK inhibitor completely blocks all MEK1/2 activity without affecting the amount of the two proteins. RNAi, however, depletes the target proteins to a large extent, but the remaining MEK1 and MEK2 is still functional and leads to a low level of constant ERK activation.

Both MEK1 and MEK2 were shown earlier to each be required for different steps during cell cycle progression. MEK1 has been shown to be required for Golgi fragmentation (32), whereas MEK2 is thought to be essential for progression through the G2/M checkpoint in cells exposed to ionizing radiation (33). MEK2-deficient cells released from a double thymidine block did indeed not progress through G2 but underwent apoptosis (data not shown).

The individual MEK1 and MEK2 knock downs each exhibited a totally different phenotype. These observations further demonstrated the respective contributions of MEK1 and MEK2 to the control of cell cycle entry via the transcriptional regulation of the ERK response genes p21cip1 and cyclin D.

Forced Activation of MEK2 Results in a Senescence-Like Phenotype due to p21cip1 Induction—The up-regulation of p21cip1 was the prominent phenotype in MEK1-deficient cells. We could show that this p21cip1 induction resulted in a senescence-like phenotype. Under normal conditions it had been shown that only MEK1 is activated in response to serum stimulation (19) and that this transient activation is sufficient to provide proliferative signals but fails to induce a sustained expression of p21cip1 and growth arrest (14). In this context we could provide evidence that MEK2 can complement for MEK1 in response to serum stimulation but causes sustained and stronger ERK activity. Although p21cip1 is a classical p53 response gene (28), the up-regulation of p21cip1 observed here, clearly dependent on sustained ERK activation as shown earlier (4), is a prerequisite for inhibition of cell proliferation and the induction of senescence.

MEK and ERK have been shown to form complexes together with scaffolding proteins like MP1 and KSR (34), where MP1 seems to have a preference for MEK1 (35). These data presented here could indicate that, indeed, MEK1 and MEK2 are part of different signaling complexes as suggested recently (36). We believe that in the MEK1 knock down situation, MEK2 might be recruited to scaffolding complexes that are normally occupied by MEK1. Because MEK2 is a more potent activator of ERK1/2 than MEK1 (25), we could show that Fra-1, which is a target of sustained ERK activity mediated by c-fos, is indeed up-regulated. Furthermore, AP1 transcription factors have been shown to promote p21cip1 transcription.

Further evidence for p21cip1 induction as a consequence of an imbalanced, MEK2-driven ERK activation resulted from the double knock down data. Here, the reduced but balanced reduction of MEK1/2 activity followed by a weak ERK activation could not reproduce the p21cip1 induction seen in MEK1-deficient cells.

Maintenance of p21cip1 levels has been linked to the inhibition of cell proliferation and the induction of senescence (30). Overexpression of p21cip1 in HCT-116 cells induced a senescence-like phenotype that could not be induced in HCT-116 p21−/− cells (37). The MEK1 knock down was indeed accompanied by the appearance of cells with a senescence-like phenotype, which could be fully prevented by the depletion of p21cip1.

However, no significant changes in proliferation could be observed within 72 h after MEK1 depletion. Because senescence is known to be established slowly, we believe that in this case the diminished colony formation in shMEK1-expressing cells is due to p21cip1 induction followed by senescence. The reported differences in the role of MEK1 in mitosis (22) are most likely attributed to the fact that HCT-116 and HT-29 cells
studied here have an ERK cascade constitutively activated by Ras and B-Raf mutations, respectively. These cell lines furthermore do not express p16INK due to promoter methylation (29). The differences do not seem to depend on the relative expression levels of MEK1 or MEK2, as HCT-116 and HT-29, which both show the same phenotype, have either MEK1 or MEK2 in excess.

**MEK2 Activity Is Required for Regulated G1/S Transition**

The MEK2 knock down resulted in a unique phenotype characterized by a delay in mitosis. ERK phosphorylation was clearly reduced in the MEK2 knock down. This suggests that MEK1 is unable to fully compensate the loss of MEK2. The observed mitotic delay was clearly linked to centrosome over-duplication followed by multipolar spindle formation and to reduced RSK activity as a consequence of the reduced ERK activity.

The phosphorylation of nucleophosmin at Thr-199 by active CDK2 during early S-phase is a critical initiator of centrosome duplication (27). Enhanced phosphorylation of nucleophosmin should lead to deregulated centrosome duplication followed by multipolar spindle formation (38). The MEK2 knock down with its multiple centrosomes and aberrant spindle phenotype exhibited a very high level of nucleophosmin phosphorylated at this specific site. Nevertheless CDK2 activity was unchanged in these cells. However, its activity was shown earlier to be dispensable for S-phase progression (39). CDK4/6 was in this context suggested to promote S-phase progression by phosphorylating the CDK2 targets Rb and nucleophosmin (39). Indeed CDK4/6 activity was highly activated due to the increased cyclin D levels, as indicated by the intense Rb phosphorylation pattern in siMEK2 cells. This increased CDK4/6 activity, thus, provides a reasonable explanation for the increase of phospho-nucleophosmin and the centrosome over-duplication. Additional evidence for a CDK2-independent mechanism came from the p21cip1 knock down experiments. The clearly increased CDK2 activity neither led to an increased nucleophosmin phosphorylation nor to centrosome over-duplication. The unchanged CDK2 activity in all knock downs may be due to the increased levels of p21cip1 and p27kip, especially 48 h after transfection. Together, MEK2 deficiency led to a somewhat contradictory phenotype. Although it led to hyperproliferative signals, a reduced proliferation rate was observed. We could show that MEK2 deficiency led to hyperphosphorylation of nucleophosmin mediated by CDK4/6. This in itself would not cause reduced proliferation. During mitosis, however, the over-

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**Fig. 7.** MEK2 depletion induces tri- or tetrapolar spindles and reduced RSK phosphorylation. HCT-116 cells, transiently transfected with the indicated siRNAs, were stained 48 h after transfection with antibodies specific for cyclin B and α-tubulin (A); DNA was visualized with 4,6-diamidino-2-phenylindole (DAPI). Cells with tri- or tetrapolar spindles are marked with arrows. B, HCT-116, transiently transfected with MEK2 siRNA, stained 48 h after transfection with antibodies specific for γ-tubulin and α-tubulin; DNA was visualized with 4,6-diamidino-2-phenylindole. Confocal microscopy was used to show multiple centrosomes (arrows) in interphase (upper panel) and mitotic cells (lower panel). The bars indicate 10 μm. C, cell lysates 48 h after transfection with the indicated siRNAs were immunoblotted with antibodies specific for RSK phosphorylated at Thr-359 and Ser-363. GAPDH served as a loading control.

**Fig. 8.** The differential role of MEK1 and MEK2 on cell proliferation. The balance of MEK1 and MEK2 signaling decides on the kinetics and intensity of ERK activation and the final outcome, i.e. proliferation or growth arrest. The delicately timed activation of ERK by MEK1, suggested to be mediated possibly by a specific MEK1-containing scaffolding complex, promotes proliferation via cyclin D-CDK4/6 activation. The sustained ERK activation by MEK2, possibly in a different complex, leads to p21cip1-mediated growth arrest. An imbalanced shift to MEK1 activity as in the MEK2 knock down leads to CDK4/6-mediated centrosome over-duplication in early S phase, aberrant spindle formation, and finally, mitotic arrest. The shift to MEK2 activity as in the MEK1 knock down leads to growth arrest and possibly enforced senescence via induction of p21cip1.
amplification of centrosomes led to a delay after the spindle assembly checkpoint.

Knock down of either MEK1 or MEK2 resulted in increased cyclin D levels. MEK2 deficiency induced increased cyclin D transcription. In contrast, the depletion of MEK1 resulted in cyclin D stabilization. This stabilization of cyclin D in the MEK1 and double knock down needs further investigation to clarify possible cross-talk between β-catenin and ERK signaling. This cross-talk is expected since GSK3/β is known to regulate the turnover of cyclin D and β-catenin (26). Furthermore, β-catenin together with T-cell factor is known to promote the transcription of cyclin D. This could explain the moderate increase of cyclin D transcription in MEK1-deficient cells. We could not elucidate the exact mechanism by which this stabilization occurs.

ERK activity has been shown to be required not only for cell cycle entry but also for progression through mitosis. The phosphorylation of RSK by ERK is required for mitotic exit (31). The obvious differences observed might be attributed to different cell types and species under these experimental conditions. The obvious differences observed might be attributed to different cell types and species used. We used human cancer cell lines with a constitutively active ERK. In contrast, Belanger et al. (21) used primary embryonic fibroblasts and T-cells. It will be of great interest to compare depletion of MEK in primary human cells to cancer cells to address possible differences, especially in the dependence on MEK signaling.

Conclusions—As summarized in Fig. 8 we could show in several cell models that the balance of MEK1 and MEK2 activities regulates the G1/S transition by appropriately modulating the kinetics and intensity of ERK activation and, thus, the transcription of p21<sup>cip1</sup> and cyclin D. The MEK1 knock down mediated a relative increase of MEK2 activity followed by sustained strong ERK activity that led to p21<sup>cip1</sup> induction and eventually to growth arrest, as observed in the colony-forming assays. When MEK1 activity became prominent as in the MEK2 knock down, the resulting ERK signaling provided proliferative signals. Serum stimulation instead preferentially induces MEK1 activity in several cell types (19). Therefore, we suggest a model in which the specific activation of either MEK1 or MEK2 is part of the cellular system that enables the mitotic switch between inhibition and enhancement of cell proliferation.

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