Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase 1<sup>act</sup>/Tubulin Interaction Is an Important Determinant of Mitotic Stability in Cultured HT1080 Human Fibrosarcoma Cells

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Abstract

Activation of the mitogen-activated protein kinase (MAPK) pathway plays a major role in neoplastic cell transformation. Using a proteomics approach, we identified α tubulin and β tubulin as proteins that interact with activated MAP/extracellular signal-regulated kinase 1 (MEK1), a central MAPK regulatory kinase. Confocal analysis revealed spatiotemporal control of MEK1-tubulin colocalization that was most prominent in the mitotic spindle apparatus in variant HT1080 human fibrosarcoma cells. Peptide arrays identified the critical role of positively charged amino acids R108, R113, R160, and K157 on the surface of MEK1 for tubulin interaction. Overexpression of activated MEK1 caused defects in spindle arrangement, chromosome segregation, and ploidy. In contrast, chromosome polyploidy was reduced in the presence of an activated MEK1 mutant (R108A, R113A) that disrupted interactions with tubulin. Our findings indicate the importance of signaling by activated MEK1-tubulin in spindle organization and chromosomal instability. Cancer Res; 70(14); OF1–11. ©2010 AACR.

Introduction

The mitogen-activated protein kinase (MAPK) signal transduction pathway plays a pivotal role in cell cycle progression, cell differentiation, and division (1–4). Deregulated MAPK signaling is a common event in tumors, and constitutive MAPK signaling transforms mammalian cells in culture (5). The canonical MAPK pathway is initiated by extracellular proliferative signals detected by membrane-tyrosine kinases, and the signal is then transduced by a cascade of phosphorylation events in the cytoplasm through Ras, Raf, mitogen-activated/extracellular signal-regulated kinase (ERK) kinase (MEK; ref. 6), and ERK (7).

The prominent role of the dual-specificity protein kinases MEK1/2 in mitosis has been extensively documented. MEK1 activity is required for normal mitotic progression, and activated MEK1 was detected in extracts from meiotic Xenopus eggs (8–10). MEK1 is also active during mitosis in mammalian somatic cells (11–14). Intriguingly, during normal G2-M in HeLa cells, MEK1 becomes activated without the corresponding activation of its substrate ERK (15). Most recently, it has been reported that MEK1/2 regulates microtubule organization, spindle pole tethering, and asymmetrical division during meiotic maturation of mouse oocytes (16). MEK1 also promotes the G2-M transition by reorganizing the Golgi apparatus in vivo (10). Furthermore, inhibition of MEK1 signaling through the expression of dominant-negative mutants, use of chemical inhibitors, or RNA interference significantly delays meiotic entry (13, 17). MEK1 inhibition causes defects in spindle formation (18, 19) and spindle checkpoint control (18, 20), as well as disorganized spindle poles, leading to misaligned chromosomes (16).

We have previously studied which of the multiple signaling pathways, downstream of the mutant constitutively active N-Ras, are required for triggering the full spectrum of phenotypic traits associated with in vitro transformation in cell culture and in vivo tumorigenicity in human fibrosarcoma cell lines. The parental HT1080 cells, harboring an endogenous mutated allele of the N-ras gene, exhibit typical features of a neoplastic transformed cell, including poor adherence, anchorage-independent growth, absence of organized actin fibers, and aggressive tumor formation in vivo. The MCH603 variants, lacking the mutant N-ras allele, have more normal growth characteristics, including a flat adherent morphology, anchorage-dependent growth, well-organized actin microfilaments, and only a weak tumorigenic potential in vivo (21). Of the various components of the MAPK pathway, only stable overexpression of a constitutively active MEK1 S218E, S222D
mutant (MEK1act; ref. 5) elicited changes resulting in an aggressive tumorigenic phenotype in MCH603 cells (21). MEK1/2 are believed to be highly specific for their downstream substrate, ERK (7). The potent biological effects of mutant MEKs, especially the constitutively active form, were explained in terms of enhanced signaling in the canonical MAPK pathway through ERK (5). It has become increasingly clear, however, that this simple linear pathway represents only a minor component of a very complex signaling circuitry (22). Our observations with MCH603MEK1act cells provided further support for the more complex role of MEK1 in tumorigenesis than had been previously envisioned. This led to the postulation that MEK1act may activate as yet unidentified target(s) outside the canonical MAPK pathway and that this activation contributes to the aggressive tumorigenic phenotype (21). Increased polyplody and multiple spindle pole defects observed in MEK1act-transfected cells would be compatible with the notion that MEK1-mediated signaling may have novel mitosis-related target(s).

In an attempt to decipher the mechanism of the apparently noncanonical transforming activity of MEK1act, we set out to identify MEK1act-interacting partners. In this report, we provide evidence that MEK1act is a tubulin-interacting protein. Interactions of MEK1act with tubulin were studied experimentally and by molecular modeling. R108A and R113A double mutation in the tubulin-binding domain negatively affected the interaction of MEK1act with tubulin and significantly compromised its effect on normal spindle function and chromosome ploidy in MCH603 cells. Our data reveal the biological relevance of the MEK1act-tubulin interaction and highlight its role in chromosomal stability.

Materials and Methods

Cell culture and transfections

The human fibrosarcoma cell line, HT1080 (containing an endogenous mutated allele of the N-ras gene), and its derivatives, MCH603, lacking the mutated N-ras gene and MCH603MEK1act (MCH603 stably transfected with the constitutively active MEK1act; ref. 21), and 293 HEK cells were selected in the presence of G418 (Invitrogen, 800 μg/mL). Cells were incubated on chamber slides (Nalge Nunc Int.) for 2 days, fixed with 2% paraformaldehyde/PBS, and treated with 0.1% Triton X-100/PBS (each 10 minutes at room temperature). Anti-β-tubulin mouse monoclonal antibody (Santa Cruz Biotechnology) and anti-MEK rabbit antibody (Cell Signaling Technology) were used at 1:50 dilution. Either Texas red-X–conjugated goat anti-mouse IgG (Invitrogen) or swine anti–rabbit-FITC (DAKO) secondary antibody was used as required at 1:200 dilution. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI; Sigma). All incubations were performed at room temperature followed by three or four washes. Slides were mounted in Prolong antifade reagent (Invitrogen-Molecular Probes) and viewed in a Zeiss LSM 510 confocal microscope equipped with a 63 × 1.4 numerical aperture. All analyses were performed as described in the Supplementary Materials and Methods and by Manders and colleagues (23).

Coimmunoprecipitation of MEK1/2 and tubulin

MCH603MEK1act cells were washed twice with ice-cold PBS and lysed in 10 mM HEPES (pH 7.2), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.02% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, and 10 μg/mL aprotinin on ice (5 min). After preclearing, the lysate (500 μg) was incubated with 30 μL of a 50% protein G-Sepharose slurry (Amersham Biosciences) for 1 hour at 4°C. The supernatant was incubated with 30 μL of anti-MEK1/2 or anti–β-tubulin rabbit antibodies (Santa Cruz Biotechnology) at 4°C overnight, then 30 μL of 50% slurry of protein A/G-Sepharose mixture (Amersham Biosciences) were added and incubated for 1 hour at 4°C. Immunocomplexes were recovered as described in the Supplementary Materials and Methods.

Peptide array binding experiments

A custom nitrocellulose-bound peptide array providing a scan of the amino acid sequence of MEK1act was obtained from Jerini Bio Tools GmbH. Spots on the array contain 13 residue peptides overlapping by 11 residues, attached to a cellulose membrane through a COOH-terminal β-(Ala)2 spacer. All procedures were carried out at room temperature. The membrane was treated with methanol, washed 3 x 10 minutes with TBS buffer [100 mMol/L KCl and 30 mMol/L Tris-HCl (pH 7.6)], and blocked with 5% nonfat milk in 0.05% (v/v) Tween 20 in TBS buffer overnight. The membrane was incubated with tubulin (25 μg/mL; Cytoskeleton Inc.) in blocking buffer with gentle shaking overnight, and nonspecific binding was removed by washing with 0.05% (v/v) Tween 20 in TBS buffer. Bound protein was transferred to a polyvinylidene difluoride membrane by electroblotting and immunodetected with anti-tubulin mouse monoclonal antibody (Santa Cruz Biotechnology; 1:200 dilution). Alanine replacement scans of MEK1 peptides 34 (HKPSGLVMARKLI), 41 (LEIKPAIRNQHIR), and 65 to 69 (GSLQVYKKAIRLGK) were carried out using similar procedures.

Flow cytometry

Trypsinized cells (5 x 10^6) were washed with PBS and incubated in ice-cold 70% ethanol/30% PBS at 4°C for 1 hour. Cells were then washed once with PBS, resuspended in 0.5 mL PBS plus 0.5 mL DNA extraction buffer [0.2 mol/L Na2HPO4,
we performed affinity purification of a crude MCH603MEK1act, eluted in the latter fraction, identified as five distinct bands specifically coimmunoprecipitated MEK1 from MCH603MEK1act and tubulin antibody precipitated and GST pull downs. MEK1 antibody specifically coimmunoprecipitated MEK1 from MCH603MEK1act cells (Fig. 2A). This shows that MEK1 and tubulin associate in vivo. Next, we mapped the tubulin-binding site of MEK1act by testing the tubulin-binding capacities of the NH2-terminal half (amino acids 1–155) bound tubulin, whereas the COOH-terminal half (amino acids 156–373) and GST only did not (Fig. 2B). GST-ERK also failed to pull down tubulin, suggesting that ERK does not directly interact with tubulin (Fig. 2C).

Actin cytoskeleton staining
Actin stress fibers were visualized by staining with fluorescein-conjugated phalloidin (Molecular Probes). Cells were plated on chamber slides, fixed in 3.7% paraformaldehyde 2 days later, treated with 0.1% Triton X-100, and then stained with phalloidin (0.005 U/μL) for 20 minutes at room temperature. Slides were mounted in ProLong fade-antifade, viewed in a Zeiss axioskop epifluorescence microscope, and images were processed by the Smart Capture II, version 2 software (Digital Scientific Ltd.).

Statistical analysis
Statistical analysis was done by paired t test with two-tailed P value. Values of P < 0.05 were considered significant.

Results
Identification of MEK1act as a tubulin-binding protein by mass spectrometry
In an effort to identify novel MEK1act-interacting proteins, we performed affinity purification of a crude MCH603MEK1act lysate on a glutathione S-transferase (GST)-MEK1act/glutathione 4B-Sepharose column. Bound proteins were eluted in two steps, first with 0.3 mol/L glycine (pH 3.0), followed by 0.1 mol/L NaHCO3 (pH 9.0). The majority of proteins were eluted in the latter fraction, identified as five distinct bands on the Coomassie-stained gel, migrating at ~110, 55, 45, 34, and 20 kDa (data not shown). Mass spectrometry of tryptic-digested proteins eluted from the 55-kDa band revealed that 13 peptides matched to β-tubulin (159 of 444 amino acids, 36%) and 6 peptides matched to α-tubulin (82 of 416 amino acid, 20%).

MEK1 colocalizes with tubulin at the centrosome, spindle, and midbody during mitosis and cytokinesis
Next, we sought to verify the MEK1-tubulin interaction experimentally. Initially, we looked into the extent of MEK1-tubulin colocalization by immunofluorescence and confocal analysis of MEK1 and tubulin in various stages of the cell cycle (Fig. 1A). Parental MCH603 cells were included as a control (Fig. 1B). Analyses of the confocal images of both MCH603MEK1act and MCH603 cells indicated that MEK1 and tubulin do not significantly colocalize during interphase (Fig. 1A–C). The values of colocalization coefficient at this stage seemed to be only 0.03 and 0.04, respectively (Fig. 1C). Additionally, Pearson’s correlation coefficient showed a lack of overlap between the green and red pixels in these cells (Supplementary Fig. S1A and B). A slight increase in the colocalization coefficient was observed in prophase in the MCH603MEK1act population, whereas MCH603 cells remained at 0.04 (Fig. 1C). The pixel distribution at this stage indicated a higher colocalization at the centrosome (Rp = 0.26; Supplementary Fig. S1A and B), compared with other regions of the cell. Interestingly, compared with interphase, the colocalization coefficient in metaphase increased to >11-fold in MCH603MEK1act and to a lesser degree in MCH603 cells (Fig. 1C). In the MCH603MEK1 act cells, almost complete colocalization was observed around the centrosome (Rp = 0.7, M1-green = 0.986, M2-red = 0.933; Supplementary Fig. S1C). As cells progressed further into anaphase and telophase, the colocalization coefficients decreased and were within the range of 0.1 to 0.3 in both cell lines (Fig. 1C), representing approximately half of the metaphase signals. As reflected in the whole-cell analyses, colocalization signals at the centrosomes were also reduced (Rp = 0.38; Supplementary Fig. S1D). In cytokinesis, the colocalization coefficients in MCH603MEK1act and MCH603 cells increased again to 0.7 and 0.45, respectively (Fig. 1C). It is worth noting that the extent of colocalization was also high in the midbody (Rp = 0.312, M1-green = 0.98, M2-red = 0.854; Supplementary Fig. S1A and E), compared with the surrounding cellular regions. The difference in the extent of colocalization in MCH603MEK1act and MCH603 cells was statistically significant in metaphase, telophase, and cytokinesis (P < 0.0087), but not in other stages of the cell cycle (Fig. 1C). In summary, the degree of colocalization of MEK1 with tubulin in mitosis increased gradually from prophase, peaking at metaphase, then decreased as cells moved through anaphase and telophase, and finally increased again at cytokinesis. In all stages of the cell cycle, the highest extent of colocalization was observed in the centrosomes in metaphase and to a lesser extent with the spindle fibers, as well as in the midbody during cytokinesis. These data indicate that MEK1-tubulin interaction is temporally and spatially regulated, and correlates with spindle organization in the mitotic phase of the cell.

We also examined the distribution of activated phospho-ERK (pERK) in these cells. Although we observed association of pERK with the centrosomes during mitosis, we did not see any appreciable association with the mitotic spindle (data not shown). Many proteins localize to the centrosome, and we did not further pursue potential interacting protein partners with pERK at this locale.
The evidence that MEK1<sup>act</sup> interacts with tubulin prompted us to test whether this interaction leads to phosphorylation of tubulin. The GST-MEK1<sup>act</sup> fusion protein in an in vitro kinase assay failed to phosphorylate tubulin, whereas ERK2 was readily phosphorylated (Fig. 2D). However, we did not investigate whether the phosphorylation of tubulin occurred in vivo. In summary, data provided by various methods support the conclusion that MEK1 interacts with tubulin in vitro and in vivo. This interaction is mediated through the NH<sub>2</sub>-terminal region of MEK1<sup>act</sup>.

Identification and characterization of tubulin-binding sites on MEK1<sup>act</sup>

To identify possible tubulin-binding sites on MEK1<sup>act</sup>, we used peptide arrays that scanned the MEK1<sup>act</sup> sequence. As shown in Fig. 3A, several potential tubulin-binding regions were identified: peptides 32 to 35 share the sequence PSGLVMA (residues 89–95), peptides 41 to 43 share the sequence PAIRNQIIR (residues 105–113), and peptides 65 to 69 share the sequence KAGRI (residues 157–161). Many of the
residues in the tubulin-binding peptides were nonpolar or basic, suggesting that hydrophobic and/or electrostatic interactions contribute to the tubulin-MEK1act interaction.

To investigate the possible role of electrostatic interactions in MEK1act-tubulin binding, we carried out "alanine scans," using peptide arrays of peptides 34 and 41, and a composite peptide corresponding to peptides 65 to 69. As shown in Fig. 3B, single alanine replacements in peptide 34 had only minor effects on tubulin binding. However, more dramatic effects were observed for peptides 41 and 65 to 69. Binding was largely lost when R108, R113, K157, or R160 were replaced by alanine, suggesting that these residues critically determine interactions with tubulin. Binding was also diminished when K156 or K168 were replaced with alanine, but the lack of requirement for these residues in the arrays shown in Fig. 3A suggests that they are not essential.

The locations of the residues shared by tubulin-binding peptides identified in Fig. 3A and the basic residues identified in Fig. 3B are shown on the crystal structure of MEK1act in Fig. 3C. Residues shared in peptides 32 to 35 and those shared in peptides 41 to 43 are positioned close to one another on the β-domain on one side of the ATP site, whereas residues shared in peptides 65 to 69 are located on the α-domain ∼40Å distant on the other side of the ATP site. Some type of structural rearrangement of MEK1 may be required for both α- and β-domains to interact with tubulin.

The critical role of basic residues of MEK1 for tubulin binding was also confirmed in pull down assays. A mutant protein, designated GST-MEK1act-Q, completely lost tubulin binding activity (Fig. 3D). This finding provides further evidence for the involvement of surface charges in MEK1 in interactions with tubulin.

Effect of GST-MEK1act on microtubule polymerization in vitro

Whether MEK1act affects the assembly of microtubules was investigated in reconstitution experiments. Here, we tested the effect of GST-MEK1act on tubulin polymerization by turbidimetric time course experiments. Microtubule formation seemed to proceed with a lag phase preceding polymerization induced by the GST-MEK1act protein, resulting in an incline shape of increasing absorbance over time (Supplementary Fig. S2A). Increased concentrations of GST-MEK1act led to an increase in the formation of microtubules, as evidenced by the higher plateau value of absorbance. In addition, transmission electron microscopic imaging has shown that microtubules

![Figure 2. Coimmunoprecipitation, GST pull down studies, and kinase assay of MEK1 and tubulin. A, total protein lysates from subconfluent MCH603MEK1act cells were coimmunoprecipitated with MEK1, or tubulin, rabbit antibodies and then tested by immunoblotting with tubulin, and MEK1, mouse monoclonal antibodies. The input proteins were included as controls. B, mapping of the tubulin-binding region in MEK1act by deletion analysis. C, characterization of ERK-tubulin interaction using GST pull down studies. D, in vitro kinase assay of GST-MEKact using tubulin and ERK as substrates.](cancerres.aacrjournals.org)
formed in the presence of GST-MEK1act are structurally similar
to those formed in the presence of Taxol (Supplementary Fig.
S2B). GST served as a negative control, confirming that the
observed effect is specific to MEK1act. Taken together, these
results show that MEK1act affects microtubule polymerization
in vitro.

Effect of MEK1act on mitotic spindle formation,
cytokinesis, and chromosome ploidy

Having established that MEK1 interacts with tubulin during
mitosis, we asked whether the presence of constitutively
active MEK1act has any effect on mitotic cells. We noted that
MCH603MEK1act cells displayed a disproportionately higher

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**Figure 3.** Peptide array binding analysis of MEK1act to tubulin. A, tubulin binding to MEK1act peptide arrays. Aligned sequences of peptides 32 to 35, 41 to 43, and 65 to 69 (with MEK1act residue numbers in parentheses) are also shown. B, binding of tubulin to MEK1act peptides having single alanine replacements. *, parent unsubstituted peptide. Arrows, reduced binding. C, location of tubulin-binding peptide regions on MEK1. The backbone structure of an N-truncated form of MEK1 [residues 61–382; (41; PDB 1s9j)] is shown as a yellow ribbon with bound ATP (red), and the inhibitor PD184352 (magenta) are included as stick models. Side chains of residues common to peptides 32 to 35, 41 to 43, and 65 to 69 are shown as space-filling spheres (white) with arginine and lysine residues colored cyan (carbon) and blue (nitrogen). The view in panel a is similar to that in Fig. 4 of ref. (41); the view in b is rotated 90° about the Y-axis. The figure was generated using Visual Molecular Dynamics (42). D, mutation of the key residues in the tubulin-binding site of MEKact results in the loss of tubulin binding. Lanes 1 and 2, the input was 5 and 10 μg of GST-MEKact, respectively. Lane 5, the input was 10 μg of GST, whereas in lane 6, the input was purified tubulin.
number of cells with aberrant mitotic spindles. Multipolar metaphase spindles were observed in 20 ± 6% of these cells, in contrast to 6 ± 1% of HT1080 and 4 ± 3% of MCH603 cells. Flow cytometry analysis confirmed the strong prevalence of polyploidy in MCH603MEK1act cells (Fig. 4A). Karyotype analysis of these cells detected several aberrant chromosome segregations (e.g., anaphase bridges) and sister chromatid separation, which are likely to cause defects in mitosis and/or cytokinesis (Fig. 4B and C). Mitotic indexes indicated that MCH603MEK1act cells are 100% polyploid, whereas HT1080 and MCH603 are mostly diploid (72–80%; Fig. 4C).

It should be noted that the level of activated MEK in the MCH603MEK1act cells is ∼3-fold higher than that seen in HT1080 cells (21). Thus, this elevated level of MEK activity may well contribute to the increased chromosomal instability of the MCH603MEK1act cells versus HT1080 cells. These data indicate that constitutive high levels of MEK activity may play a role in defective mitotic spindle formation and cytokinesis that in turn leads to chromosomal aberrations and polyploidy.

Effect of MEK1act-D mutant with disrupted tubulin-binding sites on chromosome ploidy, multiple spindles, and actin fiber organization

To test what role, if any, the MEK1act/tubulin interaction plays in determining the phenotype of cells, we transfected MCH603 cells with FLAG-MEK1act and FLAG-MEK1act-D. Western blotting confirmed that the FLAG-MEK1act-D retained its ability to activate ERK (Fig. 5A, a) and had the same kinase activity as the FLAG-MEK1act (Fig. 5A, b) but exhibited significantly decreased tubulin binding (Fig. 5A, c). Similar kinase assay and coimmunoprecipitation results were obtained when green fluorescent protein (GFP)-MEK constructs were tested (data not shown). Furthermore, colocalization coefficient analyses of MCH603-GFP-MEK1act and MCH603-GFP-MEK1act-D showed that the latter had less colocalization of MEK1act with tubulin (Supplementary Fig. S3). Therefore, we did comparative flow cytometry analysis, which revealed that MCH603 cells had 30% of 2N, 46% of 4N, and 5% of 8N, whereas MCH603-GFP-MEK1act cells had 2% of 2N, 69% of 4N, and 12% of 8N (Fig. 5B). However, MCH603-GFP-MEK1act-D cells had 20% of 2N, 50% of 4N, and 7% of 8N, a notably lower fraction of >2N cells. Karyotype analysis further confirmed these results (data not shown). Multiple spindle formation was observed in 5 ± 3% of MCH603-GFP-MEK1act-D cells (similar to MCH603 cells). In contrast, 28 ± 5% of MCH603-GFP-MEK1act mitotic cells had aberrant spindles. Thus, MEK1act/tubulin binding correlates with induction of polyploidy and multiple spindle formation.

Investigation of actin fibers by phalloidin staining also revealed dramatic differences in the cytoskeletal architecture of cells transfected with FLAG-MEK1act and FLAG-MEK1act-D. Actin fibers in MCH603-FLAG-MEK1act cells showed a highly disorganized pattern (Fig. 6), which is typical of the aggressively tumorigenic HT1080 cells (21). MCH603-FLAG-MEK1act-D cells, on the other hand, showed more organized actin fibers, similar to those in the MCH603 cells (Fig. 6).

Discussion

The critical role of the MAPK cascade in the induction of proliferation and in oncogenic transformation has been firmly established (7). One of the most convincing lines of evidence for the involvement of the MAPK cascade in oncogenic transformation came from the use of MEK1 mutants. Whereas the dominant-negative form of MEK1 reversed Ras-mediated transformation (7), the constitutively active form served as an oncogene, suggesting that the deregulation of
the MAPK cascade itself is sufficient to induce cell transformation (5).

Until recently, MEK1 was considered to be a highly selective protein, known to interact exclusively with its downstream substrate ERK (7). This led to the proposition that the potent biological effect of MEKact is the consequence of enhanced signaling in the canonical MAPK pathway through ERK (5). However, the paradigm of exclusive specificity of MEKs for ERKs is being challenged at present; for example, activation of endomembrane-associated MEK1 during normal G2-M in HeLa cells occurs without the corresponding activation of ERK (15). Most recently, it also has been reported that MEK1 regulates microtubule organization, spindle pole tethering, and asymmetrical division during meiotic maturation of mouse oocytes (16). Thus, MEK1 might be a more promiscuous protein than previously thought and some of its function could be mediated by signaling outside the canonical MAPK pathway.

In our previous work, we observed that among the various components of the MAPK pathway, only overexpression of MEK1act generated an aggressive tumorigenic phenotype in MCH603 cells (21). Several factors could contribute to the strong transforming activity of MEK1act, such as cross-talk to the c-Jun-NH2-kinase (JNK) pathway. However, MCH603 cells transfected with activated Rac, which can also activate JNK and MEK1, did not exhibit the comparable aggressive tumorigenic phenotype (21). We speculated at that time that MEK1act elicits its transforming activity through interaction with an as yet unidentified noncanonical target(s) (21).

To decipher the mechanism of the strong transforming activity of MEK1act, we used a proteomics approach to identify MEK1act-interacting proteins. Affinity purification of MCH603MEK1act and FLAG-MEK1act-D cells were immunoprecipitated with anti-FLAG M2-Affinity gel, and their kinase activity was assayed with the MEK Activity Assay kit. c, total protein lysates from subconfluent transfectants were immunoprecipitated with anti-FLAG M2 Affinity gel. The immune complexes were tested by immunoblotting with anti-tubulin and anti-MEK1 mouse monoclonal antibodies. Fold difference indicates the level of immunoprecipitated tubulin level relative to the FLAG-MEK1act-D in immunoprecipitate sample. B, ploidy of the indicated cell lines was measured by flow cytometry analysis of DNA content.

**Figure 5.** The effect of R108A, R113A double mutation (MEK1act-D) on pERK levels, kinase activity, interaction with tubulin, and ploidy. A, a, pERK in MCH603-FLAG-MEK1act and FLAG-MEK1act-D cells was assayed by immunoblotting with pERK antibody. Fold difference indicates pERK levels relative to MCH603 cells. b, total protein lysates from 293 HEK cells transiently transfected with FLAG-MEK1act and FLAG-MEK1act-D were immunoprecipitated with anti-FLAG M2 Affinity gel, and their kinase activity was assayed with the MEK Activity Assay kit. c, total protein lysates from subconfluent transfectants were immunoprecipitated with anti-FLAG M2 Affinity gel. The immune complexes were tested by immunoblotting with anti-tubulin and anti-MEK1 mouse monoclonal antibodies. Fold difference indicates the level of immunoprecipitated tubulin level relative to the FLAG-MEK1act-D in immunoprecipitate sample. B, ploidy of the indicated cell lines was measured by flow cytometry analysis of DNA content.
and a mutant in which these residues were replaced with alanine (MEKact-Q) failed to interact with tubulin in a GST pull-down assay. Examination of the crystal structure revealed that the charged arginine and lysine residues identified in this study are located on the surface of MEK. This could indicate that MEK1-tubulin interaction is dependent to some extent on ionic charges.

Tubulin and microtubules have several features that could contribute to binding positively charged amino acids on MEK1. First, α and β tubulin subunits are slightly acidic with isoelectric points between 5.2 and 5.8. Second, negatively charged amino acids (glutamic and aspartic acid) are abundantly present on the surface of the tertiary structures of α and β tubulin (data not shown). Third, microtubules are intrinsically polar hollow tubes with β tubulin at the faster polymerizing “plus” end and α tubulin at the slower polymerizing “minus” end (35). During mitosis, the minus ends of astral microtubules associate with spindle poles and are firmly anchored at the centrosome, which prevents their depolymerization (35). Several proteins, such as γ tubulin, pericentrin (36), PCM1 (37), and NuMa (38), are known to localize at the centrosome and stabilize microtubules by interacting with their minus end (32, 39). In a recent report, phospho-MEK1 was found to associate with spindle poles and cytoplasmic microtubule-organizing centers, and colocalized with centrosome proteins γ tubulin and NuMa but not with kinetochore microtubules during mouse oocyte maturation (16). Inhibition of MEK1 led to spindle pole defects, which were characterized by splaying of the minus ends of microtubules (16). Spindles in cells lacking NuMA, dynactin, or cytoplasmic dynein, which are all essential for focusing and stabilizing the spindle pole formation, had a similar morphology (38, 39). Taken together, we have established several features of MEK1, including colocalization with tubulin in the centrosome region, positively charged arginine and lysine residues in tubulin binding sites, and polymerization of tubulin in vitro, indicating that MEK1 may play a role in the regulation of microtubule organization through microtubule dynamics and stabilization.

The expression of MEK1 act profoundly affected transfected MCH603 cells and generated a phenotype shift. The most striking feature of MCH603MEK1act cells is that they are 100% polyploid, unlike the nontransfected MCH603 cells. This is consistent with abundant defects in formation of mitotic spindles in MCH603MEK1act cells. Temporal and spatial changes in the association of MEK1 with tubulin during the cell cycle suggest its role in the regulation of mitotic spindle formation, mitotic progress, and chromosome segregation. The abundance of MEK1act on the centrosome could affect interaction with tubulin and lead to aberrant spindle formation and chromosome segregation in MCH603MEK1act cells. The increased incidence of multipolar spindles in MCH603MEK1act cells might contrast with splaying of the spindle in mouse oocytes in the presence of MEK1 inhibitor. Unlike splaying and unfocused spindle poles caused by the loss of stabilization of microtubule minus ends, formation of multipolar spindles (multiple centrosomes) in MCH603MEK1act cells might be the consequence of the overstabilization of the microtubules. Multipolar spindles are induced by stabilizing drugs, such as Taxol, but not by destabilizing agents.

**Figure 6.** Effect of MEK1act and MEK1act-D on actin stress fibers. Actin stress fiber organization in indicated cell lines. Bar, 2 μmol/L.
Our data suggest not only a correlation between MEK1\textsuperscript{act}/tubulin interaction and the formation of multipolar spindles and polyploidy in MCH603MEK1\textsuperscript{act} cells, but a possible causal link as well. The MEK1\textsuperscript{act}-D mutant with disrupted tubulin binding was unable to induce polyploidy in MCH603 cells, indicating that MEK1\textsuperscript{act} requires interaction with tubulin during mitosis for aberrant microtubule organization and centrosome formation. Abnormal activity of MEK1\textsuperscript{act} could thus be a major cause of chromosome instability. We propose that polyploid chromosomes in MCH603MEK1\textsuperscript{act} cells are most likely the consequence of mitotic spindle and midbody defects caused by overstabilization of microtubules in the presence of highly expressed MEK1\textsuperscript{act} that eventually lead to incomplete mitosis and/or cytokinesis. Although MEK1\textsuperscript{act} did not phosphorylate tubulin, it did affect polymerization of tubulin dimers into microtubules \textit{in vitro}. The mechanism of how MEK1\textsuperscript{act} regulates slower tubulin polymerization \textit{in vitro} and how MEK1 and MEK1\textsuperscript{act} are involved in regulation of the balance between polymerization and depolymerization of microtubules \textit{in vivo}, as well as in regulation of the centrosome cycle, spindle checkpoint, and spindle assembly in metaphase require further study. One possibility is that spindle tubulin acts as a scaffold for MEK and provides a structural base for interaction with other, as yet unidentified, target(s).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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