Mek1 Alters Epidermal Growth and Differentiation

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ABSTRACT

The highly homologous kinases, Mek1 and Mek2, act downstream of Ras and Raf to activate extracellular signal-regulated kinase (ERK) mitogen-activated protein kinases. In epidermis, Ras and Raf promote hyperplasia; however, they act on multiple Mek-independent effectors, and the extent to which Meks can mediate these effects is unknown. To address this, we expressed inducible Meks in transgenic murine and human epidermis. Both Mek1 and Mek2 triggered ERK phosphorylation. Only Mek1, however, recapitulated Ras/Raf effects in increasing proliferation and integrin expression while suppressing differentiation, which are impacts characteristic of epidermal neoplasia. Furthermore, a kinase-dead Mek1 mutant incapable of phosphorylating ERK proteins retained ability to mediate Mek1-driven epidermal proliferation. Mek1 is thus sufficient to promote the proliferative epithelial phenotype in a manner independent of intact kinase function.

INTRODUCTION

The mitogen-activated protein kinase kinases [MAPKKs or MAP/ERK kinases (Meks)] also called MKK1 and MKK2] Mek1 and Mek2 operate downstream of Ras. Ras proteins display functional redundancy, with four canonical Ras GTPase proteins found in mammals: H-Ras, N-Ras, and two K-Ras splice variants, K-Ras4A and K-Ras4B. They signal via at least three major effector cascades initiated by binding of GTP-bound Ras to families of pathway effectors, including the Raf kinases, type I phosphatidylinositol-3 kinases (PI3Ks) and Raf guanine nucleotide exchange factors (RafGEFs; Ref. 1). These pathways operate differentially in discrete cell and tissue settings (1, 2). The specific contributions of these three major Ras effector pathways are only beginning to be studied (2), and their relative roles in tissue settings and homeostasis are unknown.

As with Ras, the three Raf isoforms, Raf1, A-Raf, and B-Raf, also exhibit features of genetic redundancy and can activate both Mek1 and Mek2. Mek1/2 are the only widely accepted Raf targets (3), and their relative roles in tissue settings and homeostasis are unknown.

MATERIALS AND METHODS

Mek1/2 Constructs. To generate Mek retroviruses, a LZRS-based retroviral construct (19) modified with a Rfa cassette from the Gateway cloning system was used as a destination vector for all coding sequences, which were first cloned into pENTR1A (Life Technologies, Inc., Carlsbad, CA). Wild-type Mek1, constitutive active Mek1, and kinase-dead (KD) Mek1 (20) were subcloned into the BamHI/EcoRI sites of pENTR1A. Wild-type Mek2, constitutive active Mek2, and kinase-dead Mek2 (21) were subcloned into the BamHI site of pENTR1A vector. To generate either constitutively active or catalytically inactive, 4-hydroxytamoxifen (4OHT)-inducible Mek proteins, the appropriate Mek cDNAs were fused at their COOH termini to a mutated ligand-binding domain of the mouse estrogen receptor α (ER-α), which fails to bind estrogen but responds to 4OHT (23). The previously generated Mek1:ER-α construct (Mek1:ER-α fused to ER-α; Ref. 24) was cut with BamHI/SalI and subcloned into the BamHI/XhoI sites of pENTR1A to generate the pENTR1A-M1E plasmid. This plasmid was used to generate inducible KD Mek1 by cloning a BamHI/BglII fragment of pSET-Mek1 into the BamHI/BglII sites of pENTR1A-M1E. The inducible KD Mek2 was generated by cloning a BamHI/BglII fragment of pSET-Mek2 into BamHI/KpnI-digested pENTR1A-M2E. For transgenic constructs, ER-α fusions of constitutively active Mek1 and Mek2 were subcloned downstream of a 2075-bp human keratin 14 promoter construct containing a 5′-intron from the β-globin gene (pBSII-K14fra; Ref. 25). All constructs were verified by sequencing before use.

Animal Studies and Cell Culture. Transgenic mice were generated on a FVB background using BsrHI-linearized constructs. The following primers were used for PCR genotyping: 5′-CGTGTGTTATTTGGGTGTC-3′ and 5′-CTCTTCTTGGCACCATTGTGGAC-3′ (for Mek1:ER) and 5′-TTGATT-GGGGATATTTGGGAAG-3′ and 5′-ATGGTAGGTTGATGTTGGAC-3′.

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(for Mek2:ER). Single transgenic F1-F3 mice were used in all experiments. To activate Mek1:ER and Mek2:ER in skin of adult mice, 1 mg of 4OHT dissolved in ethanol (10 mg/ml) was applied topically once/day to a shaved area of lower dorsal skin. Genetically matched wild-type littermates were used as controls in all experiments. Treated skin areas were harvested after either 5 days or 1 month of treatment, with the latter time point used to assess differentiation marker expression because of the ~4-week epidermal turnover cycle. For human cell culture and tissue studies, primary human keratinocytes were isolated and transduced with Mek retroviral vectors as previously described (26), with 99% gene transfer efficiency verified by immunofluorescence microscopy. Human epidermis was grafted to 6-week-old female CB.17 scid/scid mice as previously described (26), with three mice grafted and analyzed/group in duplicate independent experiments. Three to 4 weeks after surgery, grafted skin was treated with either 4OHT or ethanol control as described above.

**Protein Expression Analysis.** For immunoblotting, skin tissue and cell extract were homogenized in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 1% NP40] with protease inhibitors (Complete Mini EDTA-free, Roche, Indianapolis, IN) and phosphatase inhibitor mixture II (Sigma, St. Louis, MO). Twenty μg of protein extract were loaded/lane and subjected to 10% SDS PAGE. The following antibodies were used: rabbit anti-MEK2 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-MEK1 and mouse anti-MEK2 (Transduction Laboratories); rabbit anti-ER-α (MC-20; Santa Cruz Biotechnology); rabbit anti-phospho-p44/42 MAPK and rabbit anti-total-p44/42 MAP kinase (Cell Signaling, Beverly, MA); mouse anti-β-actin clone AC-15 (Sigma); donkey antirabbit IgG horseradish peroxidase (Amersham Biosciences, Piscataway, NJ); and sheep antio IgG horseradish peroxidase. Immunoblots were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL). For immunostaining, 7 μm of cryosections were allowed to air-dry then permeabilized with ice-cold 100% acetone for 10 min. Sections were blocked with 10% horse serum/PBS for 1 h, then incubated for 1 h at room temperature with primary antibody diluted in 2% horse serum/PBS followed by three washes with PBS and incubated for 30–60 min with secondary antibodies diluted in 2% horse serum/PBS with 2 mg/ml Hoechst. After three washes with PBS, slides were mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA) and examined under a Zeiss 100M Axio-vert microscope. Immunostaining antibodies were purchased from the following sources: rabbit anti-keratin 6 and rabbit antiomouse keratin 10 (Covance, Berkeley, CA); mouse antihuman keratin 10 (Chemicon, Temecula, CA); mouse anti-keratin 16 Ab-1 (Clone L1025; NeoMarkers, Fremont, CA), rat antimonouse Ki-67 Clone TEC-3 (Dako, Carpinteria, CA), rabbit antimonouse Ki-67 (LabVision, Fremont, CA); rat antimonouse β1 integrin (Chemicon); mouse antimonouse β1 integrin (Santa Cruz Biotechnology); rat anti-CD49f (β1 integrin; Chemicon); rabbit antimonouse involucrin (Covance); mouse antimonouse involucrin clone S45 (Sigma); rabbit anti-phospho-p44/42 MAPK (Cell Signaling); rabbit anti-ER-α (MC-20; Santa Cruz Biotechnology); and goat anti-fibrosis factor (TGF)-α (R&D Systems, Minneapolis, MN). The following antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA): Cy3 donkey antirat IgG; Cy3 donkey antigoat IgG; Cy3 donkey antirabbit IgG; and Cy3 donkey antirabbit IgG. TGF-α ELISA (Quantikine TGF-α ELISA; R&D Systems) was performed in accordance with the manufacturer’s instructions.

**Fig. 1. Expression of constitutive and regulated Mek1 and Mek2.** A, immunoblots demonstrating expression and activity of retrovectors encoding Mek1 constructs; wild-type (WT Mek1), constitutively active (CA-Mek1), kinase-dead (KD-Mek1), CA-Mek1:ER fusion (Mek1:ER), and KD-Mek1:ER fusion (KD-Mek1:ER). The specific retrovectors transduced into primary human keratinocytes in vitro are noted at the top of each lane. The specific antibodies used are noted at the right of each panel. 4OHT = 4OHT ligand for activation of human ER-α ligand binding domain (ER) fusion proteins, [−] = ethanol diluent control. B, expression and activity of retrovectors encoding Mek2 constructs; wild-type (WT Mek2), constitutively active (CA-Mek2), kinase-dead (KD-Mek2), CA-Mek2:ER fusion (Mek2:ER), and KD-Mek2:ER fusion (KD-Mek2:ER).

**Fig. 2. Activation of Mek1 but not Mek2 induces murine epidermal hyperplasia.** A, immunoblots of skin extracts from three independent CA-Mek1:ER (Mek1:ER)-transgenic murine lines (lines 11, 12, and 13; line 12 shown in all subsequent data) along with nontransgenic littermate control (LM). Actin-loading controls were performed on stripped and reprobed blots. B, immunoblots of skin extracts from three independent CA-Mek2:ER (Mek2:ER)-transgenic murine lines (lines 28, 59, and 69; line 59 shown in all subsequent data). C, skin after induction of Mek1 and Mek2 via 5 days of topical 4OHT application. Note the appearance of papules ([arrows]) and skin thickening in Mek1:ER-transgenic skin which is indistinguishable from the normal appearance seen with ethanol diluent application, which resembles the normal surface seen with ethanol diluent controls ([−]). D, induction of epidermal hyperplasia after activation of Mek1 but not Mek2. Note thickened epidermal tissue in Mek1:ER skin treated for 5 days with 4OHT compared with 4OHT-treated Mek2:ER skin, which is indistinguishable from the normal appearance seen with ethanol diluent application and in 4OHT-treated nontransgenic LM controls. Scale bars = 20 μm.
RESULTS

To examine the role of Mek1/2 in epidermal homeostasis, we generated a panel of retroviral expression vectors as well as keratin 14 promoter-driven constructs for transgenic mouse generation. Wild-type as well as constitutively active and KD Mek1/2 mutant sequences were used. To regulate Mek function with 4-hydroxytamoxifen (4OHT), active and kinase-dead constructs were fused at their COOH termini to the mutant ligand binding domain of ER-H9251. This approach has been used successfully to generate regulated Ras and Raf constructs (16, 23). Expression of all Mek constructs, either after retrovector transduction in vitro (Fig. 1, A and B) or in transgenic murine epidermis (Fig. 2, A and B), was verified by immunoblotting. Consistent with their catalytic function, constitutively active (CA) Mek1 and Mek2 both increased levels of phosphorylated ERK1/2, whereas KD mutants failed to induce this effect (Fig. 1, A and B). Cells expressing CA-Mek:ER fusions (designated Mek:ER for the remainder of this work) displayed detectable basal levels of phospho-ERK1/2 that were increased in response to 4OHT while KD-Mek:ER fusions produced no ERK phosphorylation, either with or without 4OHT (Fig. 1, A and B). Protein levels of both active and KD-Mek:ER fusion protein levels were consistently increased upon addition of 4OHT, as observed with other ER fusion proteins (16).

To examine the effects of Mek activation in epidermal tissue, we generated three independent lines of transgenic mice for both Mek1:ER and Mek2:ER. Two genotypically confirmed Mek1:ER and three Mek2:ER lines displayed detectable fusion protein expression in murine skin. Mek1 induction in murine skin promotes epidermal proliferation, increases integrin expression, and suppresses differentiation. A. Alterations in expression of epidermal proliferation, differentiation, and integrin expression accompany Mek1-induced epidermal hyperplasia. The two left columns represent nontransgenic littermate (LM) control skin treated with either ethanol diluent [-] or 4OHT, whereas the two right columns represent the same two conditions for Mek1:ER-transgenic skin tissue. Note increased immunostaining for the proliferation marker Ki-67 and the proliferation-associated keratin 6, decreased detection of the involucrin and keratin 10 differentiation markers, and increased expression of both β1 and β4 integrins multiple cell layers above the basement membrane zone. As observed with cells in culture, note increased epidermal expression of Mek1:ER protein that occurs with 4OHT treatment, as detected by antibodies to ER-α. Scale bars = 50 µm. B, mitotic index, as quantitated in Ki-67+ cells/100-µm basement membrane +/− SD from triplicate independent animals.
skin extracts (Fig. 2, A and B). Topical application of 4OHT increased levels of phosphorylated ERK1/2 in both Mek1:ER and Mek2:ER transgenics but not littermates and, in the case of Mek1:ER mice, produced clinical and histological evidence of epidermal hyperplasia in as few as 5 days (Fig. 2, C and D, and data not shown). Mek1:ER lines 12 and 13 displayed similar phenotypes, with line 12 changes more pronounced and thus chosen for more extensive study. Epidermal hyperplasia was fully established after 5 days of 4OHT and remained stable for a subsequent month of treatment, with no evidence of inflammation at either time point, as judged by lack of both inflammatory cell infiltration and increased markers such as tumor necrosis factor α and interleukin 1β (data not shown). Mek1-induced changes were entirely reversed within 10 days after 4OHT cessation, as seen with regulated Ras and Raf (16). In contrast, none of the Mek2:ER lines differed from nontransgenic controls in clinical or histological phenotype (data shown for line 59). Therefore, activating Mek1 but not Mek2 leads to hyperplasia in murine epidermis.

Induction of Ras and Raf in epidermis enhances features emblematic of the basal layer program of stratified epithelium (16). To determine the extent to which Mek1 could recapitulate these effects, we next examined markers of proliferation as well as differentiation and integrin expression following Mek1 activation. Mek1 induction increased mitotic activity and enhanced expression of the proliferation-associated keratin 6 protein. It also elevated expression of β1 and β2 integrins (Fig. 3, A and B). Epidermal differentiation markers, including involucrin and keratin 10, were suppressed (Fig. 3, A and B), although not entirely absent. No changes in cell death were observed, as judged by terminal deoxynucleotidyl transferase-mediated nick end labeling staining, and in contrast to Mek1, Mek2 induction showed no alterations in proliferation, integrins, or differentiation and was indistinguishable from untreated wild-type mice (data not shown). These findings indicate that, in murine tissue, Mek1 is sufficient to mediate the central epidermal effects of Ras and Raf in promoting the undifferentiated, proliferative program characteristic of the basal layer.

Human and murine mesenchymal cells can engage substantially different effector cascades downstream of Ras (17), so we next investigated whether Mek protein induction produced similar effects in human tissue. To do this, we used Mek retroviral expression vectors to regenerate human epidermis genetically engineered for constitutive and inducible alterations in Mek function. Retroviral transduction and human skin regeneration on immune deficient scid/scid mice were undertaken as previously described (26, 27) and resulting impacts on epidermal homeostasis examined. As observed in murine epidermis, both inducible and constitutive activation of active Mek1 in human tissue produced hyperplasia because of increased proliferation as well as augmented integrin expression and suppressed differentiation (Fig. 4A–D). Moreover, active Mek2 induction failed to alter epidermal homeostasis by any of these measures but increased detectable phospho-ERK1/2 protein expression in epidermis (Fig. 4D) as it did in transgenic mice. Mek1 effects on epidermal growth and differentiation are thus congruent in both murine and human epidermal tissues and are not merely confined to one species.

Both Mek isoforms can activate their only known targets, ERK1 and ERK2, via phosphorylation of a Thr/Glu/Tyr motif. Our observation that activation of either Mek1 or Mek2 increased detection of nuclear phosphorylated ERK1/2 in epidermis yet only Mek1 induction altered epidermal homeostasis suggested that Mek1 protein kinase activity may be dispensable for its epidermal effects. To test this possibility, we expressed constitutive and 4OHT-inducible KD Mek constructs in genetically engineered human epidermis. Topical 4OHT application increased phosphorylated ERK1/2 in Mek1:ER- and Mek2:ER-engineered human tissue but not in KD-Mek1:ER tissue (Supplementary Fig. 2) and KD-Mek2:ER tissue. Kinase-deficient Mek1, however, triggered substantial epidermal hyperplasia (Fig. 4A) in a manner similar to active Mek1, whereas KD-Mek2 did not.

**DISCUSSION**

Here, we have shown that Mek1 is sufficient to promote integral features of the undifferentiated phenotype in epidermis. Despite its
>86% amino acid sequence identity to Mek1. Mek2 exerted no effect in this setting, reminiscent of the marked differences seen in Mek1- and Mek2-knockout mice (7, 8). Although activation of both Mek proteins increased levels of phosphorylated ERK1/2, only Mek1 altered epidermal homeostasis. Furthermore, a kinase-dead Mek1 mutant also induced hyperplasia. These data indicate that simple ERK phosphorylation is not sufficient to alter epidermal homeostasis and that an intact Mek1 kinase domain is dispensable for Mek1 effects in this setting. This suggests that Mek1 signaling in epidermis proceeds via a pathway that either diverges from the canonical Ras/Raf/Mek/ERK cascade above the level of ERK MAPKs or is independent of ERK phosphorylation.

One potential mediator of Mek1 epidermal effects is TGF-α, a protein that has been implicated in Ras epidermal effects on growth, differentiation, and neoplasia (28). Although active Mek1 but not KD-Mek1 modestly increased TGF-α secretion by keratinocytes in vitro, so did Mek2, which does not drive epidermal hyperplasia (Supplementary Fig. 1A). Moreover, neither Mek1 nor Mek2 produced detectable expression of TGF-α protein in vivo in either engineered epidermis or in Mek1-transgenic mice (Supplementary Fig. 1B); therefore, TGF-α does not appear to represent a likely Mek1 effector in this setting. Examination of known Mek-interacting proteins such as KSR, MP1, and RKIP, which may act to enhance signaling specificity, may provide clues to the mechanism responsible for Mek1 impacts (29–31). Some of these proteins such as MP1 and p14 (32, 33) interact selectively with Mek1 but not Mek2 and thus constitute potential contributors to Mek1-selective effects in epidermis.

In addition to representing the major features of the undifferentiated tissue compartment of stratified epithelium, changes induced by Mek1...
are also observed in epidermal cancer, notably cutaneous squamous cell carcinoma. The finding that the Ras-MAPK cascade is up-regulated in a substantial proportion of human squamous cell carcinomas, even in the absence of primary mutations in RAS genes (27), additionally supports efforts to explore Mek inhibition in squamous cell carcinoma. Our studies provide a rationale to further explore the effects of blocking Mek1 in squamous cell carcinoma and suggest that targeting Mek2 activity may not be a viable strategy for this cancer. Mek1 but not Mek2 is thus capable of promoting the undifferentiated, proliferative program in epidermis and may represent a candidate mediator of epidermal hyperproliferation characteristic of neoplastic skin disorders.

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REFERENCES

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