ERK5 and ERK2 Cooperate to Regulate NF-κB and Cell Transformation*

Gray Pearson‡, Jessie M. English§, Michael A. White¶, and Melanie H. Cobb∥
‡the Department of Pharmacology and Cell Biology and Neuroscience, The University of Texas Southwestern Medical Center, Dallas, Texas 75390-9041
§the Department of Biological Research-Oncology, Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Abstract

We have previously demonstrated an involvement of MEK5 and ERK5 in RafBXB-stimulated focus formation in NIH3T3 cells. We find here that MEK5 and ERK5 cooperate with the RafBXB effectors MEK1/2 and ERK1/2 to induce foci. To further understand MEK5-ERK5-dependent signaling, we examined potential MEK5-ERK5 effectors that might influence focus-forming activity. Consistent with results from our focus-formation assays, constitutively active variants of MEK5 and MEK1 synergize to activate NF-κB, and MEK5 and ERK5 are required for activation of NF-κB by RafBXB. The MEK5-ERK5 pathway is also sufficient to activate both NF-κB and p90 ribosomal S6 kinase. Our results support the hypothesis that NF-κB and p90 ribosomal S6 kinase are involved in MEK5-ERK5-dependent focus formation and may serve as integration points for ERK5 and ERK1/2 signaling.

The transmission of extracellular stimuli to the nucleus is a complex process that often involves the coordinated activation of one or more three-kinase pathways, known as MAP kinase cascades (1, 2). These cascades consist of a MEK kinase, or MEKK, that phosphorylates and activates a MAP/ERK kinase, or MEK. The dual specificity MEKs phosphorylate MAP kinases on tyrosine and threonine residues, which substantially increases the activity of the MAP kinases. The active MAP kinases subsequently phosphorylate transcription factors, downstream kinases, and other substrates.

The currently known members of the MAP kinase family include ERKs 1, 2, 3, 5, and 7, and multiple c-Jun NH2-terminal kinase and p38 isoforms. ERK5, with its 816 amino acids, is

*This work was supported in part by Grants DK34128 (to M. H. C.) and CA71443 (to M. A. W.) from the National Institutes of Health and by Grants 11243 and 11414 from the Welch Foundation.
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
†To whom correspondence should be addressed: Dept of Pharmacology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9041. Tel.: 214-648-3627, Fax: 214-648-3811, mcobb@mednet.swmed.edu.
§In partial fulfillment of the requirements for the Ph.D. Supported by Pharmacological Sciences Training Grant G1907062-25 at the University of Texas Southwestern Medical Center.
¶The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; MEKK, MEK kinase; EGF, epidermal growth factor; RSK, ribosomal S6 kinase; SRE, serum response elements; Luc, luciferase; CMV, cytomegalovirus; NIK, NF-κB-inducing kinase; HbEGF, heparin-binding EGF-like growth factor; HA, hemagglutinin; GAL, galactosidase.
close to twice the size of many of the other MAP kinases (3, 4). The difference in size is because of a stretch of \(~\)400 amino acids C-terminal to the kinase domain that do not display any sequence similarity to known proteins and have no known function. On the other hand, the catalytic domain, located at its N terminus, is \(~\)50\% identical to ERK2.

ERK5 is regulated by a wide variety of mitogens and cell stresses by mechanisms that sometimes involve Ras or Src (5–12). ERK5 activity appears to be influenced by the MEK kinases MEKK3 and Tpl-2 (13, 14). Supporting one of these connections, overexpressed MEKK3 immunoprecipitated from EGF-stimulated 293 cells phosphorylates MEK5, the only known ERK5 kinase (13), in vitro. Although its activity is not sufficient to activate ERK5, Raf-1 appears to be involved in regulation of ERK5 by oncogenic Ras in 293 cells (7, 8).

Several activators of ERK5 have been identified; however, less is known about its downstream effectors. In vitro ERK5 substrates include the MADS box transcription factors myocyte enhancer factor 2A, C, and D (10, 13, 14) and the Ets class transcription factor Sap1a (9). The ability of ERK5 to activate MEF2 isoforms appears to allow it to positively regulate intracellular concentrations of c-Jun (14).

We have previously shown that increasing the activity of the ERK5 pathway by expression of MEK5DD, an activated mutant of MEK5, in NIH3T3 cells is not sufficient to stimulate the formation of foci (8). We were, however, able to uncover a role for the ERK5 pathway in focus formation when observed in a RafBXB-dependent signaling background. Foci induced by Raf-BXB are increased in number by enhancing the activity of the ERK5 pathway and are decreased in number by disruption of MEK5 or ERK5 function (8). Thus, under some conditions, MEK5 and ERK5 can impact growth and morphological transformation.

Transfection of RafBXB into cells alters the activity and localization of a large repertoire of signaling molecules including ERK1/2 (15–17). Our focus assays with RafBXB and MEK5DD suggested that Raf activates the ERK1/2 pathway to produce the synergy in focus formation observed with MEK5 and ERK5 (8). In support of this idea, we now find that MEK5DD and MEK1R4F, an active mutant of MEK1, cooperate to form foci of growth and morphologically transformed NIH3T3 cells. In addition, we explore potential mechanisms by which the MEK5-ERK5 pathway might influence focus formation. We find that MEK5DD and MEK1R4F synergize to activate an NF-\(\kappa\)B-sensitive reporter, MEK5 and ERK5 are involved in RafBXB activation of NF-\(\kappa\)B, and that the MEK5-ERK5 pathway is sufficient to activate NF-\(\kappa\)B. We also identified p90 ribosomal S6 kinase (RSK) as a target of MEK5-ERK5 signaling.

Materials and Methods

**Plasmids, Reagents, and Expression of Recombinant Proteins**

3× SRE-Luc, pCMV5-NF-\(\kappa\)B-inducing kinase (NIK), and 2× NF-\(\kappa\)B-Luc (18); heparin-binding epidermal growth factor-like growth factor (HbEGF)-Luc (19), pCMV5Myc-MEK5DD, pCMV5Myc-MEK5KM, pCMV5-RafBXB, pCMV5Myc-ERK5KM, and
pCEP4HA-ERK5CatKM (8); and pCMV5-β-GAL, pCMV5-MEK1R4F (7), PCDNA3FLAG-ERK5 (10), and pCEP4HA-RSK (20) were as described. Expression of proteins was monitored using the following antibodies: HA, 12CA5, BAbCO (Covance Research Products, Cumberland, VA), and M2 αFLAG, which were from Sigma.

**Luciferase Reporter Assays**

NIH3T3 cells were maintained as described (18). Calcium phosphate precipitates were prepared using standard protocols. For 3× SRE-Luc assays 60-mm dishes were transfected with 1 μg of 3× SRE-Luc and 1 μg of empty vector, pCMV5Myc-MEK5DD, pCMV5-MEK1R4F, pCMV5-RafBXB, pCMV5Myc-MEK5KM, or pCMV5Myc-ERK5KM as indicated. Either 4 μg of pCMV5-β-GAL or 2 μg of PRL-TK was used to control for transfection efficiency. For 2× NF-κB-Luc assays 60-mm dishes were transfected with 1.5 μg of 2× NF-κB-Luc and 1 μg of empty vector, pCMV5Myc-MEK5DD, pCMV5-MEK1R4F, pCMV5-RafBXB, pCMV5Myc-MEK5KM, pCMV5Myc-ERK5KM, or pCEP4HA-ERK5CatKM or 0.3 μg of pCMV5-NIK as indicated. 2 μg of either pCMV5-β-GAL or PRL-TK was used to control for transfection efficiency. For HbEGF-Luc assays 60-mm plates were transfected with 1 μg of HbEGF-Luc, 2 μg of PRL-TK, and 1 μg of empty vector, pCMV5Myc-MEK5DD, or pCMV5-MEK1R4F as indicated. 18–24 h post-transfection medium was replaced with Dulbecco’s modified Eagle’s medium plus 0.5% calf serum. After 24 h in low serum lysates were prepared in 0.5 ml of luciferase lysis buffer (18). Lysates were assayed for firefly luciferase and firefly Renilla activity using a dual luciferase assay kit (Promega) and the Turner Designs luminometer. Reporter gene induction was calculated by normalizing luciferase activity to either Renilla or β-galactosidase activity (18).

**Immunoprecipitations and Kinase Assays**

COS-7 cells were transfected with 1 μg of pCEP4HA-RSK and 0.5 μg of pCMV5Myc-MEK5DD and PCDNA3FLAG-ERK5 as indicated using FuGENE6 transfection reagent according to the manufacturer’s protocol (Roche Molecular Biochemicals). The appropriate amount of empty vector was used to maintain a final amount of 2 μg of DNA in each transfection. 18–24 h post-transfection, medium was replaced with Dulbecco’s modified Eagle’s medium. After 24 h without serum lysates were prepared as described previously (8). Immunoprecipitations were performed as described (7) using anti-HA antibody. Kinase assays were performed as described (8) using histone 7S as indicated.

**Focus Assays**

Focus assays were performed as described previously (21).

**Results**

**MEK5DD and MEK1R4F Synergize to Induce Growth and Morphological Transformation**

A primary mediator of Raf-dependent signaling is the MEK1/2-ERK1/2 MAP kinase cascade. However, Raf-1 coordinates the activities of signaling pathways that are independent of ERK1/2 (22). Despite the fact that Raf-1 does not apparently activate MEK5, our earlier results demonstrate that ERK5 and MEK5 are required for formation of foci of
growth and morphologically transformed cells induced by the activated Raf variant RafBXB (8). These results suggest that the ERK5 pathway is one of the other Raf-1-dependent pathways.

To define further the other Raf-mediated events that allow MEK5 and ERK5 to influence focus formation, we tested the cooperativity of ERK5 and ERK1/2 pathways to induce cellular transformation. NIH3T3 cells were transfected with vector alone, MEK5DD, an active mutant of MEK1 (MEK1R4F), or MEK5DD and MEK1R4F together. MEK5DD alone, as expected, did not induce foci. MEK1R4F clearly induced foci (Fig. 1), although much less efficiently than RafBXB (not shown; see Ref. 8). The combination of MEK5DD and MEK1R4F produced significantly more foci than MEK1R4F alone (Fig. 1). These findings indicate that the ERK1/2 and ERK5 pathways can act synergistically to induce foci of growth and morphologically transformed cells.

**MEK5DD and MEK1R4F Synergize to Activate NF-κB but Not the Serum Response Element**

SRE, integration points for ERK1/2, c-Jun NH2-terminal kinase, and p38 signaling are present in many serum-induced genes, notably that of the proto-oncogene c-Fos (23–27). The SRE is regulated by both serum response factors and ternary complex factors, including Elk-1 and Sap1a. ERK5 might influence SRE activity, because it has been shown to phosphorylate and activate Sap1a (9). It is possible then that a synergistic enhancement in the transcription of SRE-sensitive genes could occur when MEK5DD and MEK1R4F are coexpressed, which might contribute to the cooperation seen in our focus assays. We tested this possibility by transfecting NIH3T3 cells with an SRE-driven reporter and empty vector, MEK5DD, MEK1R4F, or MEK5DD and MEK1R4F together. MEK5DD neither significantly influenced the SRE reporter by itself, nor did it enhance MEK1R4F activation of the reporter (Fig. 2A). It seems unlikely then that the MEK5-ERK5 pathway influences morphological transformation through genes containing serum response elements.

NF-κB activity is influenced by MAP kinases and is required for focus formation induced by oncogenic Ras and RafBXB (28, 29). Using a luciferase reporter linked to a multimerized NF-κB-sensitive element, we tested the ability of MEK5DD and MEK1R4F to synergize in activating NF-κB family transcription factors. NIH3T3 cells were cotransfected with the NF-κB reporter and vector alone, MEK5DD, MEK1R4F, or MEK5DD plus MEK1R4F. MEK5DD was unable to activate the NF-κB-dependent reporter on its own; however, it did enhance NF-κB reporter activity stimulated by MEK1R4F (Fig. 2B). These results are consistent with the idea that MEK5DD may influence MEK1R4F-stimulated focus formation by enhancing activation of NF-κB by MEK1R4F.

**MEK5 and ERK5 Are Involved in Stimulation of NF-κB by RafBXB**

We have previously found that the MEK5-ERK5 module influences RafBXB-stimulated transformation of NIH3T3 cells. Although neither MEK5 nor ERK5 is activated directly by RafBXB, focus assays indicate that MEK5 and ERK5 participate in a subset of RafBXB-stimulated molecular events (8). Our current data indicate that the MEK5-ERK5 pathway influences NF-κB activity and, therefore, may also be involved in activation of NF-κB by RafBXB. To test this possibility we assayed the effects of MEK5KM, ERK5KM, and
ERK5CatKM (a truncated variant containing amino acids 1–451 of ERK5) on activation of the NF-κB reporter by Raf-BXB. MEK5KM and ERK5KM inhibited RafBXB activation of the NF-κB reporter (Fig. 3A). Interestingly, deletion of amino acids 452–816 renders ERK5 unable to influence NF-κB activity (Fig. 3A). Consistent with this result, ERK5CatKM does not inhibit RafBXB-induced focus formation (not shown). MEK5KM and ERK5KM do not affect the ability of the NIK (Fig. 3B) or MEKK1 (not shown) to activate the NF-κB reporter, which suggests that the ability of MEK5-ERK5 to influence NF-κB activation is pathway-specific.

Activation of the serum response element by RafBXB often correlates with focus formation (30). We therefore tested whether the expression of dominant negative MEK5 or ERK5 affects RafBXB activation of a luciferase reporter gene fused to the SRE. Neither dominant negative MEK5 nor ERK5 had an effect on the ability of RafBXB to activate the SRE-dependent reporter under conditions in which they do inhibit RafBXB activation of NF-κB (Fig. 3C). The failure of the MEK5-ERK5 kinase-dead proteins to interfere with SRE activation is a measure of their failure to block RafBXB-dependent activation of ERK1/2, as shown using a Raf mutant that cannot activate ERK1/2 (22). Based on these results we conclude that dominant negative MEK5 and ERK5 are able to inhibit a subset of Raf-BXB-stimulated transcriptional responses, such as NF-κB activation; the inhibition of NF-κB activity may be one of the factors that results in a decreased number of foci.

MEK5DD Does Not Influence the Transcription of HbEGF

In NIH3T3 cells the Raf-MEK-ERK pathway stimulates NF-κB through the production of autocrine factors, possibly HbEGF, which act on the EGF receptor (31). MEK1R4F likely utilizes a similar pathway based on epistasis. Consistent with this possibility, increases in ERK1/2 activity correlate with the production of HbEGF (32), a potential convergence point for MEK5DD and MEK1R4F signaling. We used a luciferase reporter gene linked to the mouse HbEGF promoter to monitor the effects of MEK5DD and MEK1R4F on HbEGF transcription. We found that MEK5DD did not enhance MEK1R4F-dependent activation of the HbEGF reporter (Fig. 4). Thus, the effect of MEK5DD on NF-κB activity is most likely not manifested at the level of HbEGF production.

Activation of ERK5 Is Sufficient to Increase NF-κB Activity

We and others have found that coexpression of ERK5 with MEK5DD enhances the activation of a luciferase reporter driven by GAL-MEF2C beyond that induced by MEK5DD alone (10). Thus, we tested the possibility that coexpression of ERK5 with MEK5DD may increase the activity of the NF-κB reporter. Coexpression of MEK5DD and ERK5 did stimulate a modest activation of the NF-κB luciferase reporter (Fig. 5). These findings are consistent with the conclusion that activation of ERK5 is sufficient to stimulate NF-κB.

Coexpression of MEK5DD and ERK5 Activates p90 RSK

A number of kinases, including RSKs 1–3, mitogen-activated protein kinase-associated protein kinase 2, Msk, and Mnk, are regulated by mitogen-activated protein kinase phosphorylation (1, 33–35). However, with the exception of Mnk (7), a role for ERK5 in the
regulation of these kinases has not been explored. p90 RSK has been implicated in the regulation of NF-κB (36, 37), in addition to its ability to phosphorylate effectors, such as c-Fos, (38) which might impact morphological transformation. To test the possibility that p90 RSK may be a target of MEK5-ERK5 signaling, Cos-7 cells were transfected with cDNAs encoding HA-RSK with MEK5DD alone, ERK5 alone, or MEK5DD plus ERK5. We found that coexpression of MEK5DD and ERK5 resulted in an approximately 4-fold increase in RSK activity when compared with the activity of RSK cotransfected with empty vector, MEK5DD (Fig. 6), or ERK5 (not shown).

Discussion

Proliferation and differentiation require the function of a diverse array of signaling pathways. Molecules, such as Raf, that can induce these events must coordinate the actions of multiple downstream effector pathways (15, 16). We have previously demonstrated a requirement for MEK5 and ERK5, in addition to ERK1/2, in the induction of foci by RafBXB (8). Here we show that MEK1RF4 and MEK5DD supply complementary activities to promote bypass of contact inhibition of growth. In most cases, focus formation correlates with formation of tumors in nude mice. We have explored possible mechanisms through which MEK5 and ERK5 might impact focus formation. We find that MEK5 and ERK5 are required for Raf stimulation of NF-κB, and MEK5DD synergizes with MEK1R4F to activate NF-κB. We also find that when coexpressed, MEK5 and ERK5 are sufficient for activation of NF-κB and p90 RSK.

Deregulated NF-κB activity, and abnormalities in NF-κB-encoding genes or their expression, have been identified in a number of different human tumors (39). Consistent with these clinical findings, disrupting NF-κB activity is sufficient to reduce the number of foci induced by oncogenic Ras and RafBXB in NIH3T3 cells (28, 29). NF-κB may influence tumor formation and focus formation through promoting cell survival and cell cycle entry (40, 41). ERK1/2 has been suggested to stimulate NF-κB through the production of HbEGF (31). Increasing ERK5 activity, however, has no effect on HbEGF transcription, suggesting that ERK5 may modulate NF-κB through a novel mechanism. The observation of synergy between MEK1R4F and MEK5DD in activating NF-κB further suggests that ERK5 influences NF-κB by a distinct mechanism from that used by ERK1/2.

p90 RSK activity is increased by oncogenes in cell culture systems (42, 43) and, like NF-κB may play a multifunctional role in the formation of foci. p90 RSK is involved in the stabilization of the proto-oncogene c-Fos and can inhibit apoptosis through the phosphorylation of Bad or transcription of Bcl-2 and may stimulate chromatin remodeling in a histone 3-dependent manner (42). In addition, p90 RSK phosphorylates serine 32 of IκB, a required site of phosphorylation for proteosome-mediated degradation of IκB, in response to phorbol ester treatment and overexpression of v-Src (36, 37).

Activating both ERK5 and ERK1/2, based on their known substrate profiles, broadens the range of transcription factors and other downstream effectors that may be targeted. A coordinated increase in the activity of both kinase pathways may also result in a more pronounced effect on individual signaling activities involved in focus formation than can be
generated by either pathway alone. For instance, NF-κB and p90 RSK may be integration points for two distinct activating signals. The MEK5DD and MEK1R4F activation signals could converge at a regulatory site upstream of NF-κB or alternatively, on the NF-κB homo- and heterodimers themselves. Previous reports suggest that p90 RSK can integrate the direct phosphorylation of distinct sites by ERK1/2 and phosphoinositide-dependent protein kinase to achieve a high activity state (44). The MEK5-ERK5 and MEK1/2-ERK1/2 pathways may increase p90 RSK activity through a similar integration mechanism. Further study will be necessary to discern whether MEK5DD influences MEK1R4F-dependent cellular effects by enhancing the activity of factors regulated by MEK1R4F, through activation of additional factors, or a combination of both.

Acknowledgments

We thank Jeff Cheng for contributing to an initial observation, Dale O. Henry for technical assistance, and Dionne Ware for administrative assistance. The HbEGF-Luc reporter construct was a gift from Dr. Gerhard Raab.

References

1. Lewis TS, Shapiro PS, Ahn NG. Adv Cancer Res. 1998; 74:49–139. [PubMed: 9561267]
2. English J, Pearson G, Wilsbacher J, Swantek J, Karandikar M, Xu S, Cobb MH. Exp Cell Res. 1999; 253:255–270. [PubMed: 10579927]
3. Zhou G, Bao QZ, Dixon JE. J Biol Chem. 1995; 270:12665–12669. [PubMed: 7759517]
4. Lee JD, Ulevitch RJ, Han J. Biochem Biophys Res Comm. 1995; 213:715–724. [PubMed: 7646528]
5. Abe JI, Kusuohara M, Ulevitch RJ, Berk BC, Lee JD. J Biol Chem. 1996; 271:16586–16590. [PubMed: 8663194]
6. Abe JI, Takahashi M, Ishida M, Lee JD, Berk BC. J Biol Chem. 1997; 272:20389–20394. [PubMed: 9252345]
7. English JM, Pearson G, Baer R, Cobb MH. J Biol Chem. 1998; 273:3854–3860. [PubMed: 9461566]
8. English JM, Pearson G, Hockenberry T, Shivakumar L, White MA, Cobb MH. J Biol Chem. 1999; 274:31588–31592. [PubMed: 10531364]
9. Kamakura S, Moriguchi T, Nishida E. J Biol Chem. 1999; 274:26563–26571. [PubMed: 10473620]
10. Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ, Lee JD. EMBO J. 1997; 16:7054–7066. [PubMed: 9384584]
11. Kato Y, Tapping RI, Huang S, Watson MH, Ulevitch RJ, Lee JD. Nature. 1998; 395:713–716. [PubMed: 9790194]
12. Yan C, Takahashi M, Okuda M, Lee JD, Berk BC. J Biol Chem. 1999; 274:143–150. [PubMed: 9867822]
13. Chao TH, Hayashi M, Tapping RI, Kato Y, Lee JD. J Biol Chem. 1999; 274:36035–36038. [PubMed: 10593883]
14. Chiariello M, Marinissen MJ, Gutkind JS. Mol Cell Biol. 2000; 20:1747–1758. [PubMed: 10669751]
15. Naumann U, Eisenmann-Tappe I, Rapp UR. Recent Res Cancer Res. 1997; 143:237–244.
16. Joneson T, Bar-Sagi D. J Mol Med. 1997; 75:587–593. [PubMed: 9297626]
17. Monia BP. Anti-cancer Drug Des. 1997; 12:327–339.
18. Frost JA, Swantek JL, Stippec S, Yin MJ, Gaynor R, Cobb MH. J Biol Chem. 2000; 275:19693–19699. [PubMed: 10779525]
19. McCarthy SA, Chen D, Yang BS, Garcia RJ, Cherwinski H, Chen XR, Klagsbrun M, Hauser CA, Ostrowski MC, McMahon M. Mol Cell Biol. 1997; 17:2401–2412. [PubMed: 9111309]
20. Robinson MJ, Stippec SA, Goldsmith E, White MA, Cobb MH. Curr Biol. 1998; 8:1141–1150. [PubMed: 9799732]

J Biol Chem. Author manuscript; available in PMC 2015 March 25.
21. Mineo C, Anderson RW, White MA. J Biol Chem. 1997; 272:10345–10348. [PubMed: 9099670]
22. Pearson G, Bumeister R, Henry DO, Cobb MH, White MA. J Biol Chem. 2000; 275:37303–37306. [PubMed: 11018021]
23. Gille H, Sharrocks AD, Shaw PE. Nature. 1992; 358:414–416. [PubMed: 1322499]
24. Whitmarsh AJ, Shore P, Sharrocks AD, Davis RJ. Science. 1995; 269:403–407. [PubMed: 7618106]
25. Stein B, Yang MX, Young DB, Janknecht R, Hunter T, Murray BW, Barbosa MS. J Biol Chem. 1997; 272:19509–19517. [PubMed: 9235954]
26. Gille H, Strahl T, Shaw PE. Curr Biol. 1995; 5:1191–1200. [PubMed: 8548291]
27. Treisman R. Curr Opin Cell Biol. 1996; 8:205–215. [PubMed: 8791420]
28. Finco TS, Westwick JK, Norris JL, Beg AA, Der CJ, Baldwin AS Jr. J Biol Chem. 1997; 272:24113–24116. [PubMed: 9305854]
29. Baumann B, Weber CK, Troppmair J, Whiteside S, Israel A, Rapp UR, Wirth T. Proc Natl Acad Sci U S A. 2000; 97:4615–4620. [PubMed: 10758165]
30. Kortenjann M, Thomae O, Shaw PE. Mol Cell Biol. 1994; 14:4815–4824. [PubMed: 8007980]
31. Troppmair J, Hartkamp J, Rapp UR. Oncogene. 1998; 17:685–690. [PubMed: 9715269]
32. McCarthy SA, Samuels ML, Pritchard CA, Abraham JA, McMahon M. Genes Dev. 1995:1953–1964. [PubMed: 7649477]
33. Deak M, Clifton AD, Lucoqc LM, Alessi DR. EMBO J. 1998; 17:4426–4441. [PubMed: 9687510]
34. Waskiewicz AJ, Flynn A, Proud CG, Cooper JA. EMBO J. 1997; 16:1909–1920. [PubMed: 9155017]
35. Fukunaga R, Hunter T. EMBO J. 1997; 16:1921–1933. [PubMed: 9155018]
36. Ghoda L, Lin X, Greene WC. J Biol Chem. 1997; 272:21281–21288. [PubMed: 9261139]
37. Schouten GJ, Vertegaal AC, Whiteside ST, Israel A, Toebes M, Dorsman JC, van der Eb AJ, Zantema A. EMBO J. 1997; 16:3133–3144. [PubMed: 9214631]
38. Rivera VM, Miranti CK, Misra RP, Ginty DD, Chen RH, Blenis J, Greenberg ME. Mol Cell Biol. 1993; 13:6260–6273. [PubMed: 8413226]
39. Rayet B, Gelasas C. Oncogene. 1999; 18:6938–6947. [PubMed: 10602468]
40. Mayo MW, Wang CY, Cogswell PC, Rogers-Graham KS, Lowe SW, Der CJ, Baldwin ASJ. Science. 1997; 278:1812–1815. [PubMed: 9388187]
41. Wang CY, Mayo MW, Baldwin ASJ. Science. 1996; 274:784–787. [PubMed: 8864119]
42. Nebreda AR, Gavin AC. Science. 1999; 286:1309–1310. [PubMed: 10610536]
43. Xing J, Ginty DD, Greenberg ME. Science. 1996; 273:959–963. [PubMed: 8688081]
44. Williams MR, Arthur JS, Balendran A, van der Kaay J, Poli V, Cohen P, Alessi DR. Curr Biol. 2000; 10:439–448. [PubMed: 10801415]
NIH3T3 cells plated in 5% calf serum were transfected with 500 ng of the indicated construct. Plates were scored for the appearance of foci of morphologically and growth-transformed cells 14 days post-transfection. The values represented were normalized to foci observed in cells transfected with MEK1R4F. Shown is the average of two independent experiments performed in duplicate. Error bars show S.E. A range of 5–10 foci/plate was observed for MEK1R4F-transfected cells and 50–100 foci/plate for cells transfected with MEK5DD and MEK1R4F.
Fig. 2. MEK5DD and MEK1R4F synergize to activate NF-κB but not the serum response element

A. NIH3T3 cells were transfected with the SRE reporter, along with plasmid encoding no insert, MEK5DD, MEK1R4F, or MEK5DD and MEK1R4F as indicated. Shown is the average of at least three independent experiments performed in duplicate. Relative activity refers to the increase in luciferase activity compared with cells transfected with control vector and reporter. Transfection efficiency was monitored using a Renilla firefly luciferase reporter driven by the thymidine kinase promoter. Error bars show S.E. B, same as described in A, except the activity of the NF-κB reporter was determined.
Fig. 3. MEK5 and ERK5 are required for maximal RafBXB-stimulated NF-κB activity

A, NIH3T3 cells were transfected with cDNAs encoding the NF-κB reporter, along with empty vector or Raf-BXB. Some cells were transfected with RafBXB, together with the kinase-defective mutants MEK5KM, ERK5KM, or ERK5CatKM. Shown is the average of at least three independent experiments performed in duplicate. Relative activity refers to the average luciferase activity as a fraction of that in cells expressing RafBXB. β-Galactosidase activity derived from a β-galactosidase expression vector or Renilla firefly luciferase activity was used to determine transfection efficiency. B, NIH3T3 cells were transfected with the NF-κB reporter, along with control vector or NIK. Some cells were also transfected with NIK and MEK5KM or ERK5KM. Shown is the average of at least three independent experiments performed in duplicate. Relative activity refers to the average luciferase activity as a fraction of that in cells expressing NIK. Transfection efficiency was determined as described for A. C, NIH3T3 cells were transfected with the SRE reporter, along with control
vector or RafBXB. Some cells were cotransfected with RafBXB and MEK5KM or ERK5KM. Shown is the average of at least three independent experiments performed in duplicate. Relative activity is as in A. Transfection efficiency was determined as described for A.
Fig. 4. MEK5DD and MEK1R4F do not synergize to activate the transcription of HbEGF
NIH3T3 cells were transfected with cDNAs encoding the HbEGF reporter, along with empty vector, MEK5DD, MEK1R4F, or MEK5DD and MEK1R4F as indicated. Shown is the average of at least three independent experiments performed in duplicate. Transfection efficiency and relative activity were determined as described for Fig. 2.
**Fig. 5. Coexpression of MEK5DD and ERK5 activates NF-κB**

NIH3T3 cells were transfected with cDNAs encoding the NF-κB reporter, along with control vector, MEK5DD, ERK5, or MEK5DD and ERK5 together. Shown is the average of three independent experiments performed in duplicate. Transfection efficiency and relative activity were determined as described for Fig. 2.
Fig. 6. Coexpression of MEK5DD and ERK5 activates p90 RSK
COS-7 cells were transfected with HA-RSK along with control vector, MEK5DD, or MEK5DD and ERK5 as indicated. Immune-complex kinase assays were performed on immunoprecipitated HA-RSK using histone 7S (H7S) as substrate to determine HA-RSK activity. Top panel, a representative autoradiogram from one of three independent experiments performed in duplicate. Middle two panels, immunoblots of lysates to determine HA-RSK and FLAG-ERK5 expression from a representative experiment. Bottom panel, average HA-RSK activity from three experiments performed in duplicate. RSK activity is the -fold increase in HA-RSK activity when coexpressed with the indicated cDNAs compared with the activity of HA-RSK expressed alone.