The MEK1 Proline-rich Insert Is Required for Efficient Activation of the Mitogen-activated Protein Kinases ERK1 and ERK2 in Mammalian Cells*

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MEK1 and MEK2 contain a proline-rich insert not present in any other known MEK (MAP (mitogen-activated protein)/ERK (extracellular signal-regulated kinase) kinase) family members. We examined the effect of removing the MEK1 polyproline insert on MEK activity, its binding to Raf, and its ability to activate ERKs in cells. Deletion of the insert had no effect on either the activity of MEK1 or on its ability to bind to Raf-1. Both wild type and constitutively active MEK1 coimmunoprecipitated with Raf-1 whether or not the insert was present. Deletion of the insert did not reduce activation of MEK1 by EGF or activated Raf in cells. The proline-rich insert enhanced the ability of an otherwise equally active MEK1 protein to regulate endogenous ERKs in mammalian cells. Overexpression of either constitutively active MEK1 lacking the insert or ERK2 compensates for the weaker in vivo activity of the MEK1 deletion mutant. Expression of the insert in cells reduced activation of ERKs by EGF. We conclude that the proline-rich insert is not the site of the MEK-Raf interaction and that the polyproline insert is required for its efficient activation of downstream ERKs in cells.

The MAP kinases ERK1 and ERK2 are the terminal enzymes in a three-protein kinase cascade committed to their activation (1, 2). The upstream components include the isoforms of the protein kinase Raf and the MAP/ERK kinases MEK1 and MEK2. The three kinase cascade is regulated by GTP ligation of Ras. GTP-bound Ras binds to Raf with high affinity and causes Raf translocation to the membrane where it is activated.

MEK activity was originally discovered by Ahn and Krebs by fractionating lysates of stimulated and unstimulated cells and mixed resulting fractions to identify ERK activators (3, 4). Subsequently, MEKs were purified by this group and others, and the two isoforms that activate ERK1 and ERK2 were cloned and expressed (5–8). Reconstitution experiments using purified MEK1 and MEK2 and ERK1 and ERK2 have demonstrated the quantitative activation of these MAP kinases requires only MEKs (9), although molecules that influence the kinetics of activation have been reported (10). Similarly, in vitro activation of MEK1 and MEK2 by Raf isoforms has been confirmed (11–13). Mansour et al. (14) and others identified mutations that created constitutively active forms of MEK1 and MEK2. When introduced into mammalian cells these activated MEK mutants increased ERK activity and induced effects associated with activation of the Ras/ERK pathway, such as transformation of fibroblasts, development of tumors in nude mice, and outgrowth of neurites in PC12 cells (14, 15).

The multi-enzyme MAP kinase cascade allows for amplification, because MEK1 and MEK2 are present in considerable excess of Raf. However, the MEKs and ERKs in this pathway are present at roughly equal concentrations and in some cells MEKs are in excess (16). This suggests that the MEK to ERK step exists not for amplification but for kinetic regulation (17). The presence of this step also provides additional mechanisms for sensing coincident and distinct regulatory inputs (18).

MEK1 and MEK2 contain a proline-rich insert in the C-terminal domain of the kinase catalytic core that is not present in any other known MEK family members either in higher eukaryotes or in yeast. This insert contains phosphorylation sites for proline-directed and other protein kinases and is phosphorylated in intact cells (18). In vitro, MEK1 is phosphorylated in this insert by ERKs, Cdk2, and the p21-activated kinase PAK1, among others (12, 18–20). Jelinek et al. (19) reported that a MEK1 point mutant lacking one of these phosphorylation sites no longer forms complexes with endogenous Raf. In a follow-up study they deleted the proline-rich insert from MEK1 and found that it was no longer efficiently activated in cells nor did it cause transformation (21). These data suggested that the polyproline insert of MEK1 was involved in interaction with the Ras-Raf complex, presumably through direct binding to Raf. More recently, Frost et al. (18) found that mutation of other phosphorylation sites in this proline-rich insert also reduced Raf-MEK binding, further supporting the idea that changes in the MEK1 proline-rich insert modulate coupling to Raf.

In this study we deleted the proline-rich insert of MEK1 and expressed it as a separate polypeptide with the aim of demonstrating that the insert directly binds to Raf. In addition, we examined its effects on activation of ERKs in vitro. Deletion of the proline-rich insert from a constitutively active form of MEK1, MEK1 R4F, had little effect on its in vitro protein kinase activity or its ability to coimmunoprecipitate with Raf-1, but significantly impaired its ability to activate ERKs in mammalian cells. Our findings are consistent with two conclusions. First, although phosphorylation within the proline-rich insert modulates binding of MEK to Raf, it is not the site of the interaction. Second, the polyproline insert of MEK1 is required for its efficient activation of downstream ERKs in cells.

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The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAP/ERK kinase; PCR, polymerase chain reaction; EGF, epidermal growth factor; HA, hemagglutinin; MBP, myelin basic protein; GST, glutathione S-transferase.

MATERIALS AND METHODS

Plasmids and Proteins—Recombinant His6-K52R ERK2 and active ERK2 were expressed and purified as described (9, 18). MEK1, MEK1 R4F, MEK2 R4F (kindly provided by Natalie Ahn, University of
Deletion of the Proline-rich Insert Has Little Influence on the Activity of MEK1 in Vivo—To examine the interactions mediated by the proline-rich insert, it was important to demonstrate that deletion of the insert did not influence MEK activity. Therefore, we determined the impact of the proline-rich insert on the in vitro kinase activity of MEK1. Because it had been reported that deletion of the insert prevented activation of MEK1 by Raf (21), we initially studied the properties of a constitutively active form of MEK1 in the absence of this insert. We deleted 37 amino acids (residues 265–301 of human MEK1) from the constitutively active mutant MEK1R4F and expressed MEK1R4F and MEK1R4FΔins as histidine-tagged proteins in bacteria, MEK1R4FΔins displays a marked increase in electrophoretic mobility consistent with deletion of the proline-rich domain. Recognition of MEK1R4FΔins with antibodies to both termini, along with sequencing of the mutant cDNA, confirms that the apparent size of the protein is correct and due neither to inadvertent mutations nor to proteolytic cleavage (Fig. 1A).

The activities of MEK1R4F and MEK1R4FΔins to phosphorylate and to activate ERK2 were compared using K52R ERK2 as substrate. The activities of MEK1R4F and the mutant lacking the proline-rich insert were consistently within 20% (Fig. 1, B and C). Therefore, deletion of the insert had little or no effect on the ability of MEK1 to activate ERK2 in vitro.

Deletion of the Insert Impairs the Function of MEK1R4F in Mammalian Cells—Based on their in vitro activities, we expected that both MEK1R4F and MEK1R4FΔins would cause equal activation of ERKs in cells. Their expression in transfected cells was equivalent, based on immunoblotting lysates with antibodies to both the N terminus (not shown) and the C terminus of MEK1 (Fig. 2A). The absence of the proline-rich domain was confirmed by blotting the transfected lysates with an antibody that recognizes the MEK1 insert (A2227); a signal was detected only from MEK1R4F, not from MEK1R4FΔins (Fig. 2B).

To assess the effects of these MEK mutants on activity of endogenous ERK1 and ERK2, the lysates were immunoblotted with an antiserum that recognizes both ERK1 and ERK2 and with an affinity purified antibody that selectively recognizes the doubly phosphorylated, high activity forms of ERK1 and ERK2 (23). Equal amounts of ERK1 and ERK2 were present in aliquots from each lysate (Fig. 2C). In lysates from cells transfected with MEK1R4F the anti-active ERK antibody recognized both ERK1 and ERK2, whereas in cells transfected with the control vector, neither band was visible. MEK1R4FΔins also caused a detectable enhancement of ERK1 and ERK2 activity,
The MEK1 Proline-rich Insert

Activation of endogenous ERKs by MEK1 R4F and MEK1R4FΔins. Cells were transfected with vector alone, MEK1 R4F, or MEK1R4FΔins. Immunoblot of transfected cell lysates using an antibody to the C terminus (A), the insert (B), ERK1/2 (C), and active forms of ERK1/2 (D). E, MBP kinase assay of ERK2 immunoprecipitated from lysates. F, MEK1 R4F and MEK1R4FΔins were immunoprecipitated from transfected cells and assayed as above.

Phosphorylation of the MEK1 Insert—Because the MEK1 insert contains known phosphorylation sites, we tested its phosphorylation in vitro by several protein kinases (Fig. 4). ERK2 and PAK1 phosphorylated the MEK1 insert, whereas MEKK1, MEK1, and MEK2 did not. This is consistent with phosphorylation of the intact protein on S298 by PAK1 and on T292 by ERK2 and other protein kinases (12, 18, 20, 21).

Deletion of the Proline-rich Insert Does Not Prevent Binding of MEKI to Raf-1—An original aim was to determine whether the MEK1 proline-rich insert binds to Raf-1. Therefore, we measured the ability of MEK1R4F with and without the insert to coimmunoprecipitate with endogenous Raf-1. HA-MEK1R4FΔins and HA-MEK1R4F were transiently ex-

or with ERK2, to determine whether ERK2 overexpression could overcome the reduced efficacy of MEK1R4FΔins. Transfected ERK2 was activated nearly as well by MEK1R4FΔins as by MEK1R4F (Fig. 3A). These findings support the notion that the efficiency of the activation reaction is increased in cells by the presence of the MEK1 proline-rich insert. The data further indicate that the reduced efficiency can be overcome by increasing the amount of either MEK or ERK. It is possible that the insert mediates direct binding of MEK to ERK or that it binds other proteins that affect MEK-ERK coupling. The latter is more likely, because an ERK binding site has been identified in another region of MEK1 (30). Transient transfection experiments were performed to determine whether the proline-rich insert blocked activation of ERKs by ligands. A GST fusion protein containing the 37-amino acid insert reduced the peak activation of ERK1 and ERK2 by EGF in proportion to the transfection efficiency of the cells (Fig. 3B).

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To determine whether the MEK1 insert is phosphorylated in mammalian cells in a manner that alters MEK activity, MEK1R4F and MEK1R4FΔins were immunoprecipitated from transfected cells, and their activities were compared before and after treatment with phosphoprotein phosphatase 2a. The activities of these immunoprecipitated MEK mutants were within experimental error, despite the finding that ERK activity was always greater in cells transfected with MEK1R4F (Fig. 2F). Treatment with phosphatase 2a had no effect on the activity of either MEK1R4F or MEK1R4FΔins, consistent with the lack of an activity-modifying phosphorylation of the MEK1 insert in cells under the conditions examined.

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Fig. 2. Activation of endogenous ERKs by MEK1 R4F and MEK1R4FΔins. Cells were transfected with vector alone, MEK1 R4F, or MEK1R4FΔins. Immunoblot of transfected cell lysates using an antibody to the C terminus (A), the insert (B), ERK1/2 (C), and active forms of ERK1/2 (D). E, MBP kinase assay of ERK2 immunoprecipitated from lysates. F, MEK1 R4F and MEK1R4FΔins were immunoprecipitated from transfected cells and assayed as above.

Based on immunoreactivity; however, the activation was much reduced relative to that elicited by MEK1R4F (Fig. 2D). Immunoblotting results were confirmed by measuring MAP kinase activity in lysates using MBP as substrate. MEK1R4F caused a 2.5-fold increase, whereas MEK1R4FΔins caused a 1.2-fold increase in the MBP kinase activity (data not shown). Immune complex kinase assays of ERK2 revealed similar changes in activity (Fig. 2E), with effects of MEK1R4F and MEK1R4FΔins 5-fold and less than 1.5-fold, respectively. Because the transfection efficiency was within experimental error, despite the finding that ERK activity was always greater in cells transfected with MEK1R4F (Fig. 2F). Treatment with phosphatase 2a had no effect on the activity of either MEK1R4F or MEK1R4FΔins, consistent with the lack of an activity-modifying phosphorylation of the MEK1 insert in cells under the conditions examined.

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pressed in 293 cells and immunoprecipitated with an anti-HA antibody. Antibodies against the C terminus of MEK1 were used to show that equivalent amounts of HA-MEK1R4F and HA-MEK1R4FΔIns were immunoprecipitated (Fig. 5A). Endogenous Raf-1 coimmunoprecipitated with both forms of MEK1R4F, as assessed by immunoblotting with an anti-Raf-1 antibody (Fig. 5B). To confirm that the enhanced activity of the MEK1ΔIns mutant did not account for its interaction with Raf, perhaps through a feedback phosphorylation by ERKs, we examined the ability of wild type MEK1 and MEK1ΔIns to coprecipitate with Raf-1. Again we found that Raf-1 was associated with MEK1 whether or not it contained the proline-rich insert (Fig. 5C), although wild type MEK1 bound to Raf less well than did MEK1R4F. These data suggest that the proline-rich insert is not the Raf-1 binding site. To determine whether the proline-rich insert bound to Raf in the absence of the MEK1 catalytic core, the insert–GST fusion protein was overexpressed in 293 cells. Lysates were incubated with glutathione beads. Proteins associated with the beads were separated on gels and immunoblotted with antibodies against Raf-1. Under these conditions, Raf-1 binding to the insert could not be detected (not shown).

Deletion of the Proline-rich Insert Does Not Reduce Activation of MEK1 by EGF—Because MEK1 lacking the insert coimmunoprecipitated with Raf-1, we tested the ability of wild type MEK1 with and without the insert to be activated by growth factors and Raf. Previously published work suggested that without the insert MEK1 would be poorly activated (21). Contrary to expectations, the time courses and extents of activation of wild type MEK1 and MEK1ΔIns by EGF were identical (Fig. 5D). As confirmation that the cells were equally responsive to EGF, activation of endogenous ERKs was monitored. In cells transfected with MEK1 or MEK1ΔIns, ERKs were equally activated by EGF (not shown). We also tested the ability of a constitutively activated Raf-1 mutant S259D to activate MEK1 when they were cotransfected. Again, we found that both wild type MEK1 and MEK1ΔIns were activated to a similar extent (not shown).

**DISCUSSION**

MEK1 and MEK2 contain a proline-rich insert that is not present in any of the other known MEK family members. This insert is phosphorylated in intact cells and mutation of at least two different insert phosphorylation sites diminishes the ability of MEK1 to associate with Raf-1 in cell lysates (18, 19). Furthermore, it has been reported that the insert is necessary for cell transformation (21). To understand the underlying mechanisms, we examined the effect of removing the MEK1 polyproline insert on MEK activity, its binding to Raf, and its ability to activate ERKs in cells.

We find that deletion of the insert has no effect on the activity of MEK1, either in vitro or isolated from cells, and, in contrast to earlier suggestions, removing the insert has no effect on the Raf-1 binding of MEK1. Both wild type and constitutively active MEK1 coimmunoprecipitate with Raf-1 whether or not the insert is present, indicating that the insert is not required for MEK-Raf binding. In support of this conclusion, the insert alone did not bind Raf in lysates. Apparently the phosphorylation or mutation of residues such as Thr292 and Ser308 within the insert can influence MEK1-Raf-1 binding either positively or negatively. Mutation of Thr292 to Ala inhibited the association of MEK1 with Ras-Raf complexes (19). A decrease in Raf binding was noted for MEK1 mutants with both Ser308 and Thr292 mutated to Ala (18). On the other hand, phosphorylation of Ser308 by PAK1 enhances the activation of MEK1 by Raf and growth factors, indicating that the proline insert modulates MEK1 function in cells (18).

Consistent with the lack of effect of deleting the proline-rich insert on binding of MEK1 to Raf, deletion of the insert does not reduce activation of MEK1 by EGF or activated Raf in cells. Catling et al. (21) reported that a similar MEK1 deletion protein was not activated by Raf or growth factors. Perhaps the difference in behavior of these MEK1 deletion mutants is because of the difference in the residues that were excised. The previously described MEK1 deletion mutant removed residues 270–307 (21).
We also found that the insert increases the efficiency of activation of downstream ERKs by MEK1. Factors in addition to the enzyme, MEK1, and the substrate, ERK2, apparently assist in the coupling of this kinase cascade in mammalian cells. Weber and colleagues reported that constitutively active MEK1 with the proline-rich sequence transformed fibroblasts, whereas constitutively active MEK1 without the proline insert did not (21). In that study, however, the activity of the endogenous MAP kinase was not measured. Here we show directly that the proline-rich insert unique to MEK1 and MEK2 enhances the ability of an otherwise equally active MEK protein to regulate endogenous ERKs in mammalian cells. The finding that overexpression of either MEK1R4FΔins to activate ERKs is consistent with the reduced ability of a constitutively active MEK1 mutant to regulate endogenous ERKs in mammalian cells. The finding that overexpression of either MEK1R4FΔins or ERK2 compensates for the weaker in vivo activity of the proline-rich deletion mutant suggests that the intracellular process mediated by the proline-rich insert increases the effective concentration of the substrate ERK2. The reduced activation of ERKs in cells expressing the insert alone supports this interpretation. Perhaps the insert binds to Ste5p-like scaffolding proteins that affect MEK-ERK coupling (31, 32). An alternative might be that the insert is modified or interacts with other factors in a manner that further increases MEK activity. In either case, the reduced ability of MEK1R4FΔins to activate ERKs is consistent with the reduced ability of a constitutively active MEK1 mutant with a similar but not identical deletion to transform cells (21).

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REFERENCES
1. Robinson, M. J., and Cobb, M. H. (1997) Curr. Opin. Cell Biol. 9, 180–186
2. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) Adv. Cancer Res. 74, 49–139
3. Ahn, N. G., Robbins, D. J., Haycock, J. W., Seger, R., Cobb, M. H., and Krebs, E. G. (1992) J. Neurochem. 59, 147–156
4. Ahn, N. G., Weiel, J. E., Chan, C. P., and Krebs, E. G. (1990) J. Biol. Chem. 265, 11487–11494
5. Seger, R., Seger, D., Lozeman, F. J., Ahn, N. G., Graves, L. M., Campbell, J. S., Ericsson, L., Harrylock, M., Jensen, A. M., and Krebs, E. G. (1992) J. Biol. Chem. 267, 25629–25631
6. Zhai, C.-F., and Guan, K.-L. (1993) J. Biol. Chem. 268, 11435–11439
7. Kosako, H., Nishida, E., and Gotoh, Y. (1993) EMBO J. 12, 787–794
8. Wu, J., Harrison, J. K., Dent, P., Lynch, K. R., Weber, M. J., and Sturgill, T. W. (1993) Mol. Cell. Biol. 13, 4539–4548
9. Robbins, D. J., Zheng, C.-F., and Gotoh, Y. (1993) J. Biol. Chem. 268, 5097–5106
10. Scott, A., Haystead, C. M. M., and Haystead, T. A. J. (1996) J. Biol. Chem. 270, 24540–24547
11. Gardner, A. M., Vaillancourt, R. R., Lange-Carter, C. A., and Johnson, G. L. (1994) Mol. Biol. Cell 5, 193–201
12. Mansour, S. J., Resing, K. A., Candia, J. M., Hermann, A. S., Gloor, J. W., Herskind, K. R., Wartmann, M., Davis, R. J., and Ahn, N. G. (1994) J. Biochem. (Tokyo) 116, 304–314
13. Yan, M., and Templeton, D. J. (1994) J. Biol. Chem. 269, 19067–19073
14. Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1994) Science 265, 966–970
15. Chi-Ying, F. H., and Ferrell, J. E., Jr. (1996) Trends Biochem. Sci. 21, 460–466
16. Williams, J. A., and Ferrell, J. E., Jr. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10078–10083
17. Frost, J. A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P., and Cobb, M. H. (1997) EMBO J. 16, 6426–6438
18. Jelinek, T., Catling, A. D., Reuter, C. W. M., Moodie, S. A., Wolfman, A., and Weber, M. J. (1994) Mol. Cell. Biol. 14, 8212–8218
19. Rossomando, A. J., Dent, P., Sturgill, T. W., and Marshall, D. R. (1994) Mol. Cell. Biol. 14, 1594–1602
20. Catling, A. D., Schafer, K. J., Reuter, C. W. M., Reddy, G. R., and Weber, M. J. (1995) Mol. Cell. Biol. 15, 5214–5225
21. Mansour, S. J., Candia, J. M., Gloor, K. K., and Ahn, N. G. (1996) Cell Growth Differ. 7, 243–250
22. Kobakblatchev, A., Xu, X., English, J., Wu, P., Schaefer, E., and Cobb, M. H. (1997) J. Biol. Chem. 272, 11057–11062
23. Xue, S., Robbins, D., Frost, J., Dang, A., Lange-Carter, C., and Cobb, M. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6808–6812
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Pleiman, C. M., Gardner, A. M., Johnson, G. L. (1993) Science 260, 315–319
27. Thorburn, J., Frost, J. A., and Thorburn, A. (1994) J. Cell Biol. 126, 1565–1572
28. Boulton, T. G., and Cobb, M. H. (1993) Cell Regul. 4, 357–371
29. Whitehurst, C. E., Boulton, T. G., Cobb, M. H., and Geppert, T. D. (1992) J. Cell Biol. 119, 267, 7762–7766