Constitutively Active Mitogen-activated Protein Kinase Kinase 1 (MAPKK1) and MAPKK2 Mediate Similar Transcriptional and Morphological Responses

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Abstract
Both mitogen-activated protein kinase kinase 1 (MAPKK1) and MAPKK2 function downstream of the proto-oncogene product Raf in signaling pathways that affect cell proliferation and differentiation. The isoforms were previously shown to be differentially regulated in two significant ways: (a) MAPKK1, but not MAPKK2, was phosphorylated and inactivated by the cyclin-dependent kinase p34
cdc2, and (b) p21 Ras formed a ternary complex with Raf/MAPKK1 but not with Raf/MAPKK2. To further characterize the regulation and function of the two isoforms, we compared their mode of activation by v-Mos and examined the transcriptional and morphological responses that they mediate in cultured mammalian cells. v-Mos enhanced the enzymatic activity of both isoforms to the same extent, by about 800-fold. Constitutively active MAPKK2 mutants were generated by introducing the same deletion and amino acid substitutions that have been shown to activate MAPKK1, suggesting that the conformational changes that lead to their activation are analogous. These mutants potentiated transcription from a promoter containing AP1-responsive elements and induced morphological transformation when expressed in mammalian cells, matching outcomes observed with constitutively active MAPKK1. The specific activity of p42 MAPK in the transformed cells was 3-fold higher than in cells expressing wild-type MAPKK, thereby implicating p42 MAPK as a common effector in vivo, and suggesting that sustained activation of p42 MAPK may represent a critical factor that contributes to the development of the transformed state. Altogether, the results demonstrate that the two isoforms elicit similar responses in vivo despite differences in their regulation.

Introduction
The MAP3 kinase cascade represents a conserved eukaryotic signaling module that is composed of a number of sequentially activated protein kinases, including MAPKK, MAPK, and additional kinases functioning at upstream or downstream points in the cascade (1, 2). The upstream kinases in mammalian species are Raf, Mos, and MEKK, all of which can phosphorylate and up-regulate MAPKK (3-6), whereas the downstream kinases include pp90-ribosomal S6 kinase and MAPKAP kinase-2, both of which are targeted by MAPK (7, 8). The original cascade elucidated a pathway that is growth factor regulated and was reconstituted one step at a time by using biochemical approaches to identify its components (for examples, see Refs. 9 and 10). The involvement of the cascade in regulating cell cycle progression and cell differentiation is presently well documented (11). Further studies have revealed the presence of at least five isoforms of MAPKK and MAPK in mammals and have demonstrated the existence of a number of different MAP kinase modules (12-14). It is now well established that other MAP kinase cascades mediate cellular responses to chemical, osmotic, inflammatory, or radiation-induced stress (15-18). Specific interactions among components of a particular cascade are believed to provide the required degree of singularity in signaling through these closely related pathways.

Events leading to the activation of the growth factor-regulated MAP kinase cascade are initiated at the cell membrane with the binding of ligands to cognate receptor tyrosine kinases or G-protein coupled receptors, followed by the assembly of a number of complexes within the intracellular domain of the receptor (19). One of the complexes in the case of receptor tyrosine kinases includes the growth factor receptor-bound protein GRB-2, the guanine nucleotide exchange factor SOS, the guanine nucleotide-binding protein Ras, and the serine/threonine protein kinase Raf (20, 21). Active Raf in the complex subsequently phosphorylates and up-regulates MAPKK1, the kinase that is responsible for activating p42 and p44 MAPKs. The downstream targets of this cascade include several cytosolic and cytoskeletal proteins, as well as a number of nuclear transcription factors, all

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3 The abbreviations used are: MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKK, MAP kinase kinase; MBP, myelin basic protein; SRE, serum response element; CAT, chloramphenicol acetyltransferase; FBS, fetal bovine serum; kbp, kilobase pair; CMV, cytomegalovirus.
of which are phosphorylated within minutes of growth factor treatment of quiescent cells (22). Furthermore, prolonged signaling through the pathway, due to the expression of constitutively active mutants, leads to the transformation of cells (23) and results in selective phosphorylation of chromatin components (24).

Although MAPKK1 has been clearly implicated in growth factor-induced responses, its close homologue MAPKK2 is generally believed to mediate the same responses, primarily because the two isoforms share 80% sequence identity at the amino acid level, including a unique insert of about 40 amino acids within subdomains IX and X of the kinase core. Several lines of evidence, however, indicate that the two isoforms are differentially regulated. It was first noted that the cyclin-dependent kinase p34\(^{cdcl}\) inactivates MAPKK1 upon phosphorylating threonine residues 286 and 292 in vitro, yet is unable to inactivate MAPKK2 because of the lack of homologous consensus target sites (25). In addition, MAPKK1 exhibits a significant degree of autophosphorylation within a proline-rich domain (26) that was found to be essential for Raf binding and for other regulatory functions (27). Several phosphorylated residues within this domain in MAPKK1 are absent in MAPKK2, suggesting their possible functional role, perhaps in modulating the binding of proteins that interact with MAPKK, such as phosphatases or immediate components in the kinase cascade. Indeed, characterization of the association between Ras, Raf-1, and MAPKK revealed that Raf/MAPKK1 from NIH3T3 cells formed a ternary complex with immobilized Ras, whereas the association between Raf/MAPKK2 and Ras was not detected (27, 28). The ability of MAPKK2 to complex with Raf without forming a ternary complex with Ras is consistent with the finding that although the two isoforms are up-regulated in v-Raf-transformed cells, MAPKK1 is up-regulated to a significantly higher degree in v-Ras-transformed cells (28). Threonine 292 in MAPKK1 has been implicated in promoting ternary complex formation (28), and its substitution with proline in MAPKK2 provides a rationale for the observed differences in the properties of the isoforms. This residue is constitutively phosphorylated in MAPKK1, regardless of serum conditions (27). The emerging picture is that the isoforms are differentially regulated and that they mediate responses induced by either a Ras-dependent pathway (in the case of MAPKK1) or a combination of Ras-dependent and Ras-independent pathways (in the case of MAPKK2).

To further characterize the regulation of the two MAPKK isoforms, we measured their degree of activation by the serine/threonine kinase v-Mos, and we compared the transcriptional and morphological responses that constitutively active mutants induce upon expression in cultured mammalian cells. Results from this analysis demonstrate that both isoforms elicit common responses in vivo and implicate p42 MAPK as a shared downstream effector.

**Results**

**Activation of Recombinant MAPKK2 by v-Mos.** The serine/threonine protein kinases Mos or Raf activate MAPKK1 by targeting serine residues 218 and 222 for phosphorylation (26, 29–31). These sites are found within subdomains VII and VIII of the kinase core (32) where they map to a flexible loop that has been proposed to adopt a closed conformation prior to the addition of the phosphate moiety (33–35). The kinase is inactive in its native state, but upon phosphorylation of the serine residues, the kinase becomes activated, presumably due to stabilization of the loop into a conformation that uncovers the catalytic center of the enzyme, thereby enabling the interaction between functional groups involved in catalysis and target sites on the substrate (36–38). The loop domain and the two serine phosphorylation sites are conserved in MAPKK2 (serine residues 222 and 226; Fig. 1), but given that the sequence of the two isoforms diverges at 20% of the amino acid residues (39–41), it was not self-evident that Mos can activate MAPKK2 to the same degree as MAPKK1. To address this point, the activity of wild-type MAPKK2 was determined after incubation with immunoprecipitated v-Mos (Table 1). v-Mos enhanced the activity of MAPKK2 by about 600-fold, comparable to the degree of activation of MAPKK1 (Table 1; Ref. 42). This finding indicates that the recognition of the two isoforms by v-Mos is most likely identical and implicates serine residues 222 and 226 in MAPKK2 as putative regulatory phosphorylation sites.

**Constitutively Active Mutants of MAPKK2.** Serine residues 222 and 226 were substituted with aspartic acid to mimic the negative charges and structural rearrangements that are introduced upon phosphorylation, with the goal of generating a mutant of MAPKK2 that is locked in the active-state conformation. This strategy was successfully used to generate activated mutants of MAPKK1 (31, 42, 43).
The specific activity of the bacterially expressed MAPKK2(S222D/S226D) mutant was 200 times higher than the wild-type kinase, as measured by the phosphorylation of p42 MAPK(T183A) (Table 1) or p42 MAPK(K52R) (data not shown). To test whether mutants displaying a higher specific activity of MAPKK2 could be engineered, we took advantage of the finding that the deletion of residues 32 to 51 from MAPKK1 resulted in a 25-fold enhancement of basal activity and that this deletion synergized with the regulatory site point mutations (42). We found that deletion of residues 44 to 51 from MAPKK1 was sufficient to induce a 60-fold increase in basal activity; therefore, we deleted the corresponding residues from MAPKK2 (residues 48 to 55; denoted as ΔN4 in Fig. 1), resulting in a mutant that was nine times more active than the wild-type kinase (Table 1). The ΔN4 truncation and the two serine-to-aspartate point mutations synergized in enhancing the specific activity of MAPKK2, yielding a mutant that was 2100 times more active than wild-type kinase (Table 1). All of these mutants induced the activation of wild-type p42 MAPK toward its substrate MBP (Fig. 2), thereby indicating that they can efficiently interact with the activation loop of p42 MAPK to target the two regulatory residues, threonine 183 and tyrosine 185, for phosphorylation (44).

**Potentiation of Transcription from AP1-responsive Elements.** Oncoproteins such as v-Ras, v-Raf, v-Mos, or constitutively active MAPKK1 potentiate transcription from reporter constructs containing a DNA regulatory region known as the AP-1-responsive element or the 12-O-tetradecanoylphorbol-13-acetate response element (42, 45, 46). This cis-acting region affects the expression of immediate-early genes by recruiting a heterodimeric protein complex composed of the transcription factors Fos and Jun (47, 48). Recent evidence indicates that mitogens trigger the transactivation potential of the AP-1 complex in part by stimulating the activity of several MAPK isoforms (46, 49, 50). Members of the MAP kinase family have also been shown to mediate the induction of fos gene expression by phospho-

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Unpublished results.
Fig. 3. Activation of AP-1 in cells expressing constitutively active MAPKK2. A, cells were cotransfected with an AP-1-regulated CAT reporter plasmid along with a construct that drives expression of the indicated MAPKK variant from a CMV promoter. A control transfection (Vector) was performed in the presence of the reporter plasmid and the parent CMV vector. CAT activity in 1 μg of soluble cell extract was measured and normalized to values obtained from transfections with the parent or wild-type MAPKK constructs. The baseline value matched in all cases and showed insignificant fluctuations in two independent repeats of the experiment. The relative increase in CAT activity was also variable in extracts containing activated MAPKK. B, posttranslational modification of p42 MAPK in 20 μg of soluble extract was evaluated after SDS-PAGE and Western blot analysis. Unmodified p42 MAPK exhibited normal gel migration and is labeled p42. Phosphorylated p42 MAPK displayed a retarded migration and is labeled p42*. Note that the amount of p42* is higher in extracts containing JN4/S222D/S226D than in S222D/S226D extracts.

Promote cell transformation (42, 55, 56), we examined whether constitutively active MAPKK2 would be capable of inducing the same phenotype. The outcome could not have been predicted a priori since the Raf/MAPKK2 complex exhibits differing properties than Raf/MAPKK1 (27, 28), with the likelihood that a Ras-independent pathway preferentially targets Raf/MAPKK2.

Infection of NIH3T3 cells with retroviruses encoding hemagglutinin-tagged, constitutively active MAPKK2 resulted in the formation of highly refractile and rounded cells within 48 h (Fig. 4C). The morphological transformation was evident prior to selection with hygromycin and was more pronounced during and after completion of selection (Fig. 4D). In contrast, expression of the wild-type isoform of MAPKK2 (Fig. 4, A and B) induced no changes when compared to mock-infected cells or to cells infected with the parent vector. The phenotypically transformed cells could not be distinguished by morphological criteria from rounded cells undergoing mitosis or apoptosis. Their growth behavior upon prolonged culture without passage resulted in the formation of hundreds of transformed foci surrounded by rounded as well as spindle-shaped cells that were no longer contact inhibited and that grew in disorganized patterns (data not shown). Cells expressing activated N-Ras(Q61K) (Fig. 4E) or constitutively active MAPKK1 (Fig. 4F) also showed similar kinetics of appearance of morphological transformation. A minor fraction of the cells appeared normal after selection in all transfections, including those with N-Ras(Q61K). Since our analysis was restricted to mass-infected cells, such phenotypically normal cells may have shut off expression of transduced genes or may have reverted by a desensitizing mechanism.

Kinase assays were performed on p42 MAPK immunoprecipitated from transformed cells expressing either of the activated MAPKK mutants, from normal cells expressing wild-type exogenous MAPKK, and from cells growing in 10% FBS. We invariably found that p42 MAPK was three times more active in extracts from transformed cells than in extracts from normal cells. The 3-fold increase in specific activity closely matches the 4.8-fold increase that was detected in N-Ras(Q61K) extracts and mirrored a relatively higher amount of p42 MAPK that migrated more slowly by SDS-PAGE (data not shown). This result suggests that sustained activation of p42 MAPK contributes to the development of the transformed state and indicates that the activities of both MAPKK isoforms are tightly regulated in normal cells.

Discussion

We have demonstrated in this report that immunoprecipitates of v-Mos activate recombinant MAPKK2 comparably to MAPKK1. This result complements recent findings that the activation of MAPKK1 and MAPKK2 in REF-1 rat cells takes place in concert with the conditional expression of v-Mos at the permissive temperature (57). That v-Mos is directly involved in the activation of the MAPKK2 isoform is now further strengthened with our in vitro assays. In addition, since Mos was recently shown to interact with MAPKK1 in the two-hybrid system (58), it is unlikely that an intermediate exists between Mos and MAPKK, supporting the notion that Mos can directly interact with either isoform of MAPKK in catalyzing their activation.

Other upstream kinases, including members of the Raf family and MEKK, can also activate MAPKK1 and MAPKK2 (27, 59, 60), thereby suggesting that both MAPKK isoforms are focal points for converging signaling pathways. The physiological significance of such redundant functions may be relevant during developmental stages when either of the activators is expressed or during cell cycle periods when only one of them is found in its active state. Added onto this variability is the possibility that each MAPKK isoform may itself be differentially expressed since the available sequence in their promoter elements are not conserved (39, 61).

Our finding regarding the activation of both MAPKK isoforms by similar lesions suggests that analogous structural changes have occurred. The means by which the enzymes adopted an activated conformation upon substituting the phosphorylated serine sites with negatively charged residues may have been mediated by the added carboxyl groups, which are proposed to take part in reconstituting salt bridges that are needed to stabilize the loop in an open conformation.
Fig. 4. Morphological transformation of NIH3T3 cells expressing activated MAPKK2. NIH3T3 cells were infected with retroviruses carrying wild-type MAPKK2 (A and B), activated MAPKK2-ΔN4/S222D/S226D (C and D), activated N-Ras(Q61K) (E), or activated MAPKK1(ΔN4/S218E/S222D) (F). Photographs were taken 2 days after infection, before selection against uninfected cells (A and C), or 9 days after infection, when cells were grown in the presence of hygromycin for 1 week (B and D-F). Foci were prevalent only in cells expressing the activated mutants (data not shown). Two different lines of NIH3T3 cells yielded similar results.

(33). As for the nature of the activation that is induced by the ΔN4 truncation, we note that it occurs independently of enhancing the rate of autophosphorylation of the two regulatory serine residues since the activation is observed when the serines are substituted with alanines (data not shown) or with the negatively charged residues. It is unclear why the truncation was more effective in MAPKK1 than in MAPKK2 (60-fold versus 9-fold); however, since the primary sequence at the amino terminus of the isoforms exhibits significant divergence, the variability in the extent of activation may be a consequence of different folding patterns in this region. We also found that the MAPKK2(ΔN4/D47G) mutant, containing the truncation along with an aspartic acid to glycine substitution (D47G), was four times more active than MAPKK2(ΔN4) (Table 1). The combination of these two lesions could have plausibly facilitated folding of the affected region into a more active configuration rather than affecting protein stability.

The potentiation of AP-1 transcriptional activity was found to be mediated by either isoform of constitutively active MAPKK. This result supports the view that MAPKK1 and MAPKK2 share overlapping physiological functions, either as intermediates in the same signaling pathway or as components of parallel pathways that mediate the activation of a common set of downstream nuclear factors, including Fos and Jun. Our findings also implicate p42 MAPK as a putative mediator of the transcriptional response, yet we do not rule out the likelihood that other members of the MAP kinase family may actually be involved in this process (62). Since p42 MAPK has been recently shown to modulate transcription from the SRE in the promoter of the fos gene (50, 63), and since expression of constitutively active MAPKK1 also enhanced SRE-mediated transcription (56), the observed activation of AP-1 in our experiment may be explained by an increased synthesis of the Fos protein and by the phosphorylation of both Fos and Jun at sites that up-regulate transcription.

Our experiments also revealed that expression of constitutively active MAPKK2 in NIH3T3 cells results in their morphological transformation. This finding underscores the importance of regulatory networks that modulate the activity of
the wild-type isofom. Not much is known about the in vivo regulation of MAPKK2 except that it is activated after treatment of serum-starved cells with platelet-derived growth factor (28). It is unclear whether a Ras-independent pathway mediates this activation, or whether Raf-1 or other members of the Raf family are necessarily involved, but given that Ras does not interact in vitro with the Raf/MAPKK2 complex (27, 28), a potentially novel signaling pathway that is independent of Ras might be implicated in the pathway leading to MAPKK2. Our results imply that such a signaling pathway plays a role in regulating cell growth and cell cycle progression. The physiological significance of the Raf/MAPKK2 complex is also unsubstantiated at present, but if it is functionally relevant, then a number of schemes that lead to the activation of MAPKK2 could be envisioned. The simplest one invokes the activation of Raf in a Ras-dependent or independent manner, followed by the association of Raf and MAPKK2 in a complex that excludes Ras, and the phosphorylation and release of MAPKK2. A structural component may be needed to sequester Raf/MAPKK2 from Ras.

The activity of p42 MAPK was 3-fold higher in cells transformed by activated MAPKK2 than in normal cells, implicating p42 MAPK as a downstream target in vivo. Sustained activation of p42 MAPK is also observed in cells transformed with activated Ras or MAPK1 and, therefore, represents a common factor that may contribute to the transformation of NIH3T3 cells.

Materials and Methods

Bacterial Expression Constructs and Protein Purification. Construct pKK-D was designed to express hexahistidine-tagged, wild-type MAPKK2 under control of the T7 promoter in the bacterial strain BL21(DE3)pLysS (Novagen). To generate pKK-D, the 1.2-kbp fragment encoding wild-type human MAPKK2 was recovered from pQEX-MEK2 [kindly provided by Dr. K-L. Guan (University of Michigan, Ann Arbor, MI)]. Ref. 39] by digestion with BamHI and was subcloned into the BamHI site of pRSET-A (Invitrogen). The resulting fusion protein contains a 4,000-mer with the sequence MQSGSHHHHHHHGASMTRGGNGRGDLDYDDDDKDRWGS added to the amino terminus of MAPKK2.

Site-directed mutagenesis using single-stranded DNA, the Mutagen kit (Bio-Rad), and appropriate primers were used to introduce mutations in the coding region of pKK-D. An account describing this approach has been outlined previously (64). Single-stranded DNA was derived from pKK-D and was used as a template in producing the S222D/S236D MAPKK2 construct. To generate the ΔN4 truncation, the two Kst sites that map within the gene were mutated without affecting the encoded amino acids, and two new Kst sites were introduced at codons 47/48 and 55/56. This manipulation yielded construct pMT1, which encodes for MAPKK2 containing three amino acid substitutions: D47G, E48A, and E55G. pMT1 was subsequently digested with KstI to remove 24 bp, and the ends of the resulting 4.1-kbp DNA fragment were ligated under intramolecular conditions, yielding construct pMT2W, which encodes for MAPKK2 that lacks amino acid residues 48 to 55 but still contains the D47G substitution. The construct encoding ΔN4 MAPKK2 was finally generated after reintroducing the aspartate codon at position 47 by site-directed mutagenesis on pMT2W single-stranded DNA. ΔN4/S222D and ΔN4/S222D/S236D expression constructs were also generated by starting with pMT2W single-stranded DNA. All targeted regions were sequenced to confirm the outcome of mutagenesis.

Conditions for the expression and purification of recombinant MAPKK2 are identical to those described for MAPK1 (64). Recombinant p42 MAPK was prepared as described previously (65).

Kinase Assays and Activity Measurements. Recombinant wild-type MAPK1 and MAPK2 were each activated in vitro by v-Mos immuno-purified from Swiss 3T3 (Taq-7) cells as described (26). Activation of reactions were carried out at 30°C for 3 h and included 12 μg/ml MAPKK, 0.1 mM ATP, 10 mM MgCl2, 20 mM HEPES (pH 7.4), 2 mM DTT, and 0.01% Triton X-100. Supernatants from the reactions were then diluted into an assay mixture (total volume 2 μl) containing 20 mM HEPES, 2 mM DTT, 0.01% Triton X-100, 10 mM [γ-32P]ATP (2000 cpm/ml), 1 μM p42 MAPK (T183A), and a final concentration of 0.1 μg/ml MAPKK. Reactions were carried out at 30°C, and time points were sampled at 2, 4, and 6 min by quenching 25 μl of the assay mixture with Laemml sample buffer. p42 MAPK (T183A) was resolved by SDS-PAGE, and the incorporated radiolabel was quantified by Phosphorimager analysis. Control reactions were carried out in parallel using nonactivated MAPKK at a final concentration of 0.5 μg/ml. The basal activities of constitutively activated MAPKK2 mutants were measured under standard reaction conditions, at MAPKK2 concentrations ranging from 0.025–0.5 μg/ml. Under the conditions described, all reaction rates of p42 MAPK phosphorylation were linear. Duplicate time courses were carried out for each condition, and rates were determined from linear least squares regression of the six time points. Rates of p42 MAPK autophosphorylation, measured in the absence of MAPKK, were subtracted from each rate measurement.

The phosphorylation and activation of wild-type p42 MAPK (160 ng) by MAPKK2 (20 ng) was carried out under standard assay conditions in the presence of [γ-32P]ATP in a final volume of 23 μl. The mix was incubated at 30°C for 2 min, after which 2 μl of 2.5 mg/ml MBP (Sigma Chemical Co.) were added. Reactions were subsequently quenched at 2-min intervals, and the proteins were resolved by SDS-PAGE (3.5/15% gels), followed by quantifying the radiolabel in MBP by Phosphorimager analysis.

To assay for MAPK activation in NIH3T3 cells, p42 MAPK was immunoprecipitated from 40 μg of soluble extract using antibody SC-154 (Santa Cruz Biotechnology) coupled to protein A-Sepharose CL-4B (Pharmacia). After incubation for 2 h in the presence of extract buffer (50 mM β-glycerophosphate (pH 7.4), 0.25 mM Na2VO4, 1.5 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin, and 1 mM benzamidine) containing 0.1% Triton X-100, the beads were washed three times with extract buffer plus 0.1% Triton X-100 and once with extract buffer plus 0.02% Triton X-100. Kinase reactions (40 μl) were performed as described above in the presence of 7 μM MBP, 2.5 μM protein kinase A inhibitor peptide, 2.5 μM protein kinase C inhibitor peptide, and 25 μM calmidazolium (66). The reactions were terminated after 4 min to remain linear versus time. Under these conditions, only 2% of MBP was phosphorylated using extracts containing the highest level of p42 MAPK activity.

Transient Expression and CAT Assays. Constructs for transient expression of MAPKK2 are based on the pMCL vector, which drives gene expression from a CMV promoter and which appends a hemagglutinin tag sequence at the amino terminus of the produced protein (42). Constructs were generated by digesting the bacterial expression constructs with HindIII, followed by partial digestion with BamHI, and subsequent ligation of the 1.2-kbp fragment with pMCL that had been digested with BamHI and HindIII.

Electroporation of the 293 cell line (67) was carried out using 10 μg of pMCL/MAPK2 and 5 μg of the reporter plasmid 10XAPI-CAT [kindly provided by Drs. M. Karin and A. Aronheim (University of California, San Diego, La Jolla, CA)]. Since the 293 cell line is cold sensitive, cells were not placed on ice prior to electroporation. After electroporation, cells were allowed to recover in the cuvette for 15 min at room temperature, after which they were seeded onto 6-cm plates in the presence of DMEM+10% FBS. The media was replaced after 1 day with DMEM supplemented with either 0.1 or 10% FBS. Cells were washed with PBS (pH 7.1) 48 h after transfection, and extracts were collected using the lysis buffer supplied with the CAT Enzyme Assay System kit (Promega). CAT assays were performed in the presence of [14C]chloramphenicol (Du Pont/NEN), N-butyryl CoA, and 1 μg of soluble protein. Reactions were terminated after 15 min at 37°C to remain in the linear range of the assay. The amount of butyrylated chloramphenicol was determined by scintillation counting after extracting the mix once with xylene and back-extracting twice with 0.25 mM Tris (pH 8.0).

Retroviral Vectors and Infection of NIH3T3 Cells. The retroviral vector pCTV08 [kindly provided by Dr. Robert Kay (University of British Columbia, Vancouver, British Columbia, Canada); Ref. 68] was modified to yield pMT3A by removing the BamHI and HindIII sites that flank the hypogymcin phosphohydrolase gene and reintroducing them into a novel stuffer that contains, in order, a unique BgIII site, a ribosomal binding site.
sequence, a start codon, a sequence encoding for the hemagglutinin tag, a unique BamHI site, a unique Affili site, a unique HindIII site, and a Sall site. This fragment replaced the stuffer in pCTV68 (68). The MAPK/K1 and MAPK/K2 genes were subcloned into the BamHI and HindIII sites of pMTV3A. The construct encoding activated N-Ras(G61K) was kindly provided by Dr. R. Kay. The construct encoding for β-galactosidase was generated after recovering the gene from pSV-bGal (Promega) following digestion with Drai and HindIII, blunt-ending with Klenow, and ligating the fragment with pCTV68 that had been digested with Sall and blunt-ended. All of the constructs were propagated in the bacterial strain MC1061/P3 in the presence of ampicillin and tetracycline.

Viral stocks were obtained by transfecting the BOSC23 cell line following established guidelines (69) and by using a detailed protocol kindly provided by Dr. Martin Scott. Transfection efficiency was about 15–20% as estimated by using the β-galactosidase construct and staining the BOSC23 cells with X-gal. NIH3T3 cells (8 × 10^5), seeded onto a 10-cm plate, were infected with 3 ml of virus stock in the presence of 8 µg/ml polybrene for 5 h, after which DMEM plus 10% FBS was added. Selection with hygromycin (0.2 mg/ml) was initiated 48 h later, with periodic changes of the culture medium every 3 days. Cells were not passaged during the course of the experiment.

Extracts were collected by washing cells twice with ice-cold PBS and once with extract buffer, after which the cells were scraped and briefly sonicated over ice. Extracts were centrifuged for 10 min before storing the supernatants in aliquots at −80°C.

Western Blot Analysis. Soluble protein extracts (20 µg) from transiently transfected 293 cells or from infected NIH3T3 cells were resolved by SDS-PAGE (3.5/10% gels), electrotransferred to Immobilon P (Millipore), and probed with the monoclonal antibody 12C85 (recognizing the HA tag) or with the polyclonal antibody SC-154 (recognizing p42 MAPK) and to a lesser degree p44 MAPK (Santa Cruz Biotechnology).

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