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Mitogen-activated protein kinases $p42^{mapk}$ and $p44^{mapk}$ are required for fibroblast proliferation

(protein kinase cascade/kinase antisense/dominant negative mutants/growth control/H^+-killing selection)

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ABSTRACT **The mitogen-activated protein kinases (MAP kinases) $p42^{mapk}$ and $p44^{mapk}$ are serine/threonine kinases rapidly activated in cells stimulated with various extracellular signals by dual phosphorylation of tyrosine and threonine residues. They are thought to play a pivotal role in integrating and transmitting transmembrane signals required for growth and differentiation. Here we demonstrate that activation of these ubiquitously expressed MAP kinases is essential for growth. To specifically suppress MAP kinase activation in fibroblasts, we transiently expressed either the entire $p44^{mapk}$ antisense RNA or $p44^{mapk}$ kinase-deficient mutants (T192A or Y194F). As expected, and through independent mechanisms, both approaches strongly inhibited MAP kinase activation. The antisense reduced the expression of endogenous $p42^{mapk}$ and $p44^{mapk}$ by 90%, whereas overexpression of the T192A mutant inhibited growth factor activation of both endogenous MAP kinases by up to 70%. As a consequence, we found that the antisense as well as the T192A mutant of $p44^{mapk}$ inhibited growth factor-stimulated gene transcription (collagenase promoter assay with chloramphenicol acetyltransferase reporter) and cell growth. These effects were proportional to the extent of MAP kinase inhibition and reversed by coexpression of the wild-type $p44^{mapk}$. Therefore we conclude that growth factor activation of $p42^{mapk}$ and $p44^{mapk}$ is an absolute requirement for triggering the proliferative response.**

Mitogen-activated protein kinases (MAP kinases), also described as extracellular signal-regulated kinases (ERKs), belong to a group of protein-serine/threonine kinases that are activated in response to various stimuli (growth factors, neurotransmitters, differentiating agents, heat shock) in virtually all cell types (see refs. 1–4 for reviews). Two highly related mammalian MAP kinases, $p44^{mapk}$ and $p42^{mapk}$, also called ERK1 and ERK2, have been cloned and found to be ubiquitously expressed in vertebrates (5–9) and highly homologous to the yeast kinases SLT2 (10), KSS1 (11), and FUS3 (12). A unique feature of this family of protein kinases is that they require dual phosphorylation on both tyrosine and threonine residues to become fully active (13). The sites of phosphorylation, identified in $p42^{mapk}$ (14) and conserved in all members of the family, were found to reside on a Thr-Glu-Tyr sequence of the kinase subdomain VII. Recently a MAP kinase activator was purified, cloned, and shown to be a "dual-specificity" kinase able to phosphorylate and reactivate recombinant $p42^{mapk}$ and $p44^{mapk}$ (refs. 15–19; G.P., A. Brunet, G.L., and J.P., unpublished results). This MAP kinase activator, now referred to as MAP kinase kinase, could be phosphorylated and activated *in vitro* by v-Raf or constitutively active forms of c-Raf (20–22). Thus, the protooncogene product Raf-1 appears to be one of the upstream members of the kinase cascade transmitting growth factor signals.

As far as growth control is concerned, Raf-1 certainly plays a central role. Two major arguments support this view: (*i*) constitutively active forms of Raf are oncogenic (23) and (*ii*) expression of *raf-1* dominant negative alleles reverses *ras* transformation and inhibits cell growth (24). Does MAP kinase also play such a critical role in the control of cell growth? This question remains to be answered. If the kinase cascade is linear, MAP kinases should be as critical as Raf-1; however, it is more likely that a branched signaling network is going to be discovered, and therefore the function and the role of each element of the cascade will have to be established separately. We previously demonstrated that synergistic mitogens synergize at the level of MAP kinase activation (25) and that sustained activation of MAP kinase during G_1 progression appears to be essential for triggering entry into the S phase of the cell cycle (25, 26). Here, exploiting the use of either MAP kinase antisense or dominant negative alleles, we directly demonstrate that MAP kinase activation is essential for G_0-arrested fibroblasts to enter the cell cycle.

MATERIALS AND METHODS

Materials. Highly purified human α-thrombin (3200 NIH units/mg) and human recombinant basic fibroblast growth factor (FGF) were generous gifts of J. W. Fenton II (New York State Department of Health, Albany) and D. Gospodarowicz (University of California Medical Center, San Francisco), respectively. [γ-^{32}P]ATP and the enhanced chemiluminescence (ECL) immunodetection system were from Amersham; bovine myelin basic protein (MBP) and bovine serum albumin were purchased from Sigma; Triton X-100 was from Pierce. αI Cp42 and αII Cp42 are two rabbit polyclonal antisera raised against a C-terminal peptide of rat $p42^{mapk}$ corresponding to the sequence IFEETARFQPGYRS (27). Monoclonal antibody 12CA5, raised to a peptide from influenza hemagglutinin HA1 protein (28), was purchased from Babco (Emeryville, CA).

Cell Culture. The established Chinese hamster lung fibroblast line CCL39 (American Type Culture Collection), its derivative PS120, which lacks Na^+/H^+ antiporter activity (29), and corresponding transfected cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, from GIBCO/BRL) containing 7.5% fetal bovine serum, penicillin (50 units/ml), and streptomycin sulfate (50 μg/ml). Unless otherwise stated, growth-arrested cells were obtained by total serum deprivation for 16–24 hr.

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Abbreviations: MAP kinase, mitogen-activated protein kinase; CAT, chloramphenicol acetyltransferase; MBP, myelin basic protein; FGF, fibroblast growth factor.

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Stable and Transient Transfection. For stable transfection, CCL39 cells (10^6 per 10-cm plate) were transfected with 15 μg of pcDNA-neo expression vector ($p44^{mapk}$ cDNA constructs) by the calcium phosphate coprecipitation technique (30). After 48 hr of culture the medium was replaced with DMEM/1% fetal bovine serum with neomycin analogue G418 at 500 μg/ml. After an additional 2 days, the medium was changed to the final selective medium, containing 7.5% fetal bovine serum and G418 at 500 μg/ml.

For transient expression, a H^+-killing selection technique was employed. PS120 cells (5×10^5 per 6-cm plate, unless otherwise indicated) were cotransfected by the calcium phosphate technique with 1 μg of pEAP expression vector (Na^+/H^+ antiporter cDNA; refs. 31 and 32) and 10 μg of pcDNA-neo expression vector ($p44^{mapk}$ cDNA constructs). Forty-eight hours after transfection, cells were subjected to an acid-load selection that killed nontransfected cells, usually >90% of the cell population (31, 32). Expression levels or MBP kinase activities were measured the next day in surviving cells.

DNA Constructs and Expression Vectors. The cloning, construction, and expression of the epitope-tagged $p44^{mapk}$ (pcDNA-neo vector; Invitrogen, San Diego) were previously reported (9). The Thr → Ala and Tyr → Phe substitutions respectively at positions 192 and 194 (T192A, Y194F) were introduced into the Chinese hamster $p44^{mapk}$ cDNA by the method of Kunkel (Bio-Rad mutagenesis kit). $p44^{mapk}$ antisense constructs were derived from the epitope-tagged $p44^{mapk}$ cloned into the pcDNA-neo vector. Restriction fragments (*Sac* I–*Bgl* II, 240 bp; *Sac* I–*Kpn* I, 600 bp; *Sac* I–*Hin*dIII, 850 bp; *Sac* I–*Sac* I, 1750 bp) were subcloned in the reverse orientation in the pECE vector (33). The *Sac* I site of the small fragments is within the cytomegalovirus promoter; the second *Sac* I site is in the polylinker of the pcDNA-neo vector.

Western Blot Analysis. Transfected cells were washed twice with cold phosphate-buffered saline and lysed in Triton X-100 lysis buffer [1% (wt/vol) Triton X-100/50 mM Tris·HCl, pH 7.5/100 mM NaCl/50 mM NaF/5 mM EDTA/40 mM β-glycerophosphate/200 μM sodium orthovanadate with phenylmethanesulfonyl fluoride (0.1 mM), leupeptin (1 μg/ml), and pepstatin A (1 μM)] for 25 min at 4°C. Insoluble material was removed by centrifugation at 12,000 × *g* for 15 min at 4°C. Proteins from cell lysates were separated in SDS/7.5% polyacrylamide gels and electrophoretically transferred to Hybond-C Extra membranes (Amersham) in 25 mM Tris/192 mM glycine. Membranes were blocked in Tris-buffered saline (20 mM Tris·HCl, pH 7.5/137 mM NaCl) containing 3% nonfat dry milk. The blots were then incubated with antiserum αII Cp42 (1:1000) or monoclonal antibody 12CA5 (1:500) in blocking solution for 2–4 hr at 25°C washed in Tris-buffered saline, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000, Sigma) or goat anti-mouse IgG (1:1000, Sigma) in blocking solution for 1 hr. The blots were visualized by the Amersham ECL system.

Immune-Complex Kinase Assay. Cell lysates were prepared as described above and incubated for 3 hr at 4°C with either 2 μl of antibody 12CA5 (hemagglutinin epitope) or 2 μl of anti-MAP kinase antibody (αI Cp42) preadsorbed to protein A-Sepharose. Immune complexes were washed four times with Triton X-100 lysis buffer and 1 time with kinase buffer (20 mM Hepes, pH 7.4/10 mM $MgCl_2$/1 mM dithiothreitol/10 mM *p*-nitrophenyl phosphate). MBP kinase activity was assayed by resuspending the final pellet in a total volume of 40 μl of kinase buffer containing MBP (0.25 mg/ml) and [γ-^{32}P]ATP (50 μM; specific activity, ≈5500 cpm/pmol). Reactions were initiated with ATP. After incubation at 30°C for 10 min, assays were terminated by the addition of 40 μl of 2× Laemmli sample buffer. The samples were heated at 95°C for 5 min and analyzed by SDS/12% PAGE. The gels were then stained with Coomassie blue, dried, and subjected to autoradiography. Phosphate incorporation was measured by excising substrate bands from the gel and counting the radioactivity by liquid scintillation.

Other Methods. Chloramphenicol acetyltransferase (CAT) assays were performed as described (34). Protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce) with bovine serum albumin as standard. SDS/PAGE was performed with the buffer system of Laemmli (35). Prestained molecular weight markers were from Bio-Rad.

RESULTS

MAP Kinase Dominant Negative Mutants. "Wild-type" and point mutants of $p44^{mapk}$, tagged with an influenza hemagglutinin epitope, were placed under the control of the cytomegalovirus promoter and stably expressed in Chinese hamster lung fibroblasts (CCL39 cell line). Mutations that abrogate phosphorylation at the key regulatory sites (T192A and Y194F) of the cloned $p44^{mapk}$ (9) abolished kinase activity as revealed by the lack of response to serum growth factors (Fig. 1). A similar result was previously shown for murine $p42^{mapk}$ (7). Analyses of neomycin-resistant (neo^R) colonies revealed readily measurable expression of the $p44^{mapk}$ active construct (1- to 5-fold the level of endogenous $p44^{mapk}$) and a somewhat lower expression of the kinase-deficient constructs (decrease in the level of expression and in the number of colonies). Although this was the first indication suggesting that the $p44^{mapk}$ kinase-deficient mutant may exert a dominant-negative growth effect, independent clones expressing either the wild-type or the mutated kinase did not show differences in the sensitivity of the growth factor response. Median effective doses (ED_{50} values) for serum-induced thymidine incorporation were identical for all $p44^{mapk}$ alleles expressed (data not shown).

We therefore set up a transient transfection assay to markedly overexpress both the active and inactive forms of $p44^{mapk}$ and to investigate the incidence of the transgene on the activation of endogenous MAP kinases. We cotransfected PS120, a Na^+/H^+ antiporter-deficient derivative of CCL39 cells (29), with either of the $p44^{mapk}$ constructs and a plasmid bearing the Na^+/H^+ antiporter gene (31). Two days after transfection, application of an acid-load selection eliminated >90% of the cells (31, 32) and specifically enriched for efficiently transfected cells. Under these conditions both the "wild type" and the kinase-deficient $p44^{mapk}$ mutant were expressed at very high and similar levels (Fig. 2*A*). Note that

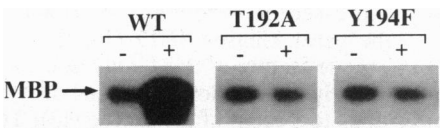

FIG. 1. MBP kinase activities of wild-type and mutated forms of $p44^{mapk}$ following stable transfection in hamster fibroblasts. MBP kinase activity of stably transfected $p44^{mapk}$ wild type (WT) and mutated forms (T192A and Y194F). Chinese hamster lung fibroblasts (CCL39 cell line) stably transfected with the epitope-tagged forms of $p44^{mapk}$ were arrested in G_0 by serum starvation for 16 hr. Equal amounts of extract proteins from resting cells (−) or cells stimulated with 10% fetal bovine serum for 5 min (+) were immunoprecipitated with the monoclonal antibody 12CA5 directed against the influenza hemagglutinin epitope. MBP kinase activity in these immunoprecipitates was measured as described in *Materials and Methods*. Note that the two mutated forms of $p44^{mapk}$ fail to respond to serum. In parallel, cell extracts were analyzed by SDS/PAGE followed by immunoblotting with the 12CA5 antibody to ensure that the $p44^{mapk}$ hemagglutinin-tagged proteins were indeed expressed to identical levels (data not shown).

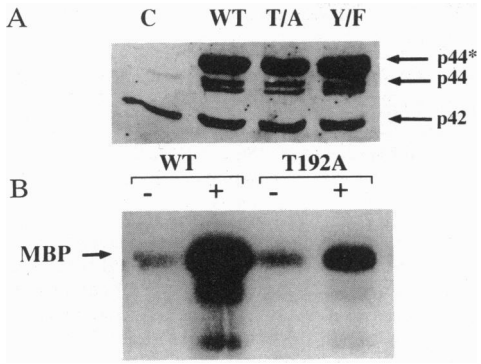

FIG. 2. Level of expression and MBP kinase activities of wild-type and mutated forms of $p44^{mapk}$ following transient transfection in hamster fibroblasts. (*A*) Transient expression in PS120 cells of the wild-type and mutated forms of $p44^{mapk}$. Cell extract proteins were separated by SDS/PAGE and immunoblotted with the αII Cp42 antiserum, which recognizes both MAP kinase isoforms (27). Cells were transfected with the vector alone (control, C) or with the epitope-tagged $p44^{mapk}$ wild type (WT) or mutated forms, T192A (T/A) and Y194F (Y/F). Note the overexpression of the epitope-tagged $p44^{mapk}$ isoform (p44*). An extra band appears in the p44 region that corresponds to a proteolytic cleavage of the overexpressed p44*. (*B*) Growth factor activation of the endogenous $p42^{mapk}$ following overexpression of wild-type and mutated $p44^{mapk}$. Parallel cell extracts derived from the experiment described in *A* were incubated with αI Cp42 antiserum, which specifically immunoprecipitates p42mapk (27), and MBP kinase activity was determined. Extracts were from serum-starved cells (−) or from cells stimulated for 5 min with α-thrombin (1 unit/ml) and basic FGF (25 ng/ml) (+). Cells were starved for 5 hr before growth factor stimulation.

addition of the epitope tag facilitated detection of transfected $p44^{mapk}$ by slightly decreasing gel mobility. To avoid interference with the transfected $p44^{mapk}$, we measured activation of endogenous MAP kinase by using antibodies that specifically immunoprecipitate $p42^{mapk}$ (27). Thrombin and FGF together stimulate $p42^{mapk}$ activity about 10-fold as assayed with MBP as substrate. This growth factor response was specifically blunted (up to 70%) by the expression of $p44^{mapk}$ T192A mutant (Fig. 2*B*). Although variable in absolute value, this inhibition was observed in five independent experiments with the two kinase-deficient forms of $p44^{mapk}$ but not with the wild-type form (inhibition varied from 40% to 70%).

Expression of $p44^{mapk}$ Antisense Suppresses Endogenous MAP Kinase Activity. In an alternative approach to reduce endogenous MAP kinase activation, but using the same transient transfection assay (acid-load selection), we expressed $p44^{mapk}$ antisense RNA. The antisense construct specifically reduced, in a dose-dependent manner, the expression of both endogenous isoforms of MAP kinases. The highest dose tested (20 μg of antisense plasmid) reduced the expression of $p44^{mapk}$ and $p42^{mapk}$ by 90% (Fig. 3*A*). As a consequence, and in contrast to the sense RNA, $p44^{mapk}$ antisense expression suppressed growth factor activation of MAP kinases (Fig. 3*B*). Coexpression of both the antisense and sense RNA construct reversed the inhibition, pointing out the specificity of the antisense action (data not shown).

Inhibition of MAP Kinases Inhibits Growth Factor-Induced AP1 Activity. Having established two independent means for specifically blunting growth factor activation of MAP kinases, we then analyzed the consequences on growth factor-induced gene transcription and cell proliferation. Thrombin or FGF, two growth factors for CCL39 cells (36), stimulated the basal activity of the collagenase promoter (Fig. 4*A*). Interestingly, expression of the $p44^{mapk}$ T192A mutant drastically suppressed growth factor-stimulated transcriptional activity. Expression of the wild-type form of $p44^{mapk}$ was

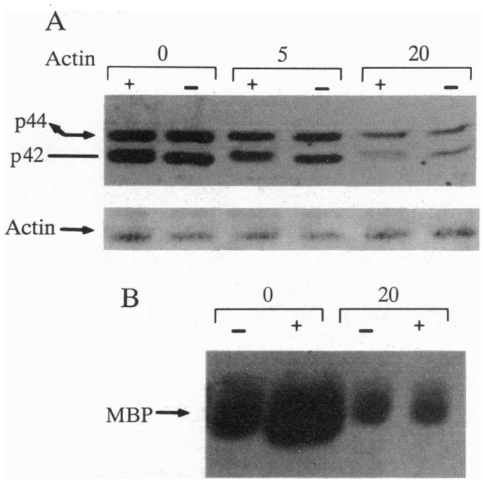

FIG. 3. Transfection of $p44^{mapk}$ antisense inhibits expression of both MAP kinase isoforms. (*A*) Immunoblotting with anti-MAP kinase antiserum (αII Cp42) of proteins from cells transfected with DNA carrier alone (lanes 0), 15 μg of DNA carrier plus 5 μg of $p44^{mapk}$ antisense vector (lanes 5), or 20 μg of $p44^{mapk}$ antisense vector (lanes 20). Prior to lysis cells were serum-starved for 5 hr (−) or stimulated with growth factors (+) as in Fig. 2*B*. Actin was visualized by staining the gel before electrotransfer. Quantitation of the levels of $p42^{mapk}$, determined by densitometry scanning, were 75% and 95% reduction, respectively, for 5 μg and 20 μg of antisense vector transfected. (*B*) Measurement of MBP kinase activity in immunoprecipitates of endogenous $p42^{mapk}$ from cells transfected with carrier DNA (lanes 0) or with 20 μg of $p44^{mapk}$ antisense vector (lanes 20). Cells were stimulated (+) or not (−) with growth factors as in *A*. Transient transfections were carried out as described in *Materials and Methods*. PS120 cells (3×10^6 per 10-cm dish) were cotransfected with 4 μg of pEAP vector together with either 0, 5, or 20 μg of $p44^{mapk}$ antisense vector adjusted to 20 μg with empty vector. In this experiment the 1750-bp antisense construct was used.

capable of reverting, in a dose-dependent manner, the inhibitory effect of the $p44^{mapk}$ T192A mutant (Fig. 4*B*). Similar results were obtained by the expression of the $p44^{mapk}$ antisense RNA (data not shown).

Inhibition of MAP Kinases Suppresses Cell Growth. To analyze the effects of MAP kinase inhibition on cell proliferation, we counted the colonies that emerged 1 week after the transient transfection assay and acid-load selection. Expression of either of the two $p44^{mapk}$ mutants, T192A or Y194F, significantly reduced colony number (Table 1). This inhibitory effect was dependent on the amount of plasmid transfected, was observed in three independent experiments, and apparently was more pronounced with the T192A mutant. Consistent with these results, we observed, in resting cells transiently expressing the $p44^{mapk}$ T192A and Y194F mutants, a 50–70% inhibition of serum-induced reinitiation of DNA synthesis measured by thymidine incorporation (data not shown). In a similar experiment, we transiently transfected either $p44^{mapk}$ sense or different antisense RNA constructs. All the antisense constructs, independent of their size, were very potent in their growth-inhibitory action (Table 2). Here again the growth-inhibitory effect was reverted by cotransfection of the $p44^{mapk}$ sense RNA, pointing to a specific effect rather than a general toxic action.

DISCUSSION

In this report we have illustrated two approaches to specifically reduce MAP kinase activity in response to mitogenic stimuli. Both approaches required a high level of expression of the antagonizing agent. In the case of $p44^{mapk}$ antisense expression, the mechanism of suppression is most probably via the formation of RNA duplexes sensitive to rapid degra-

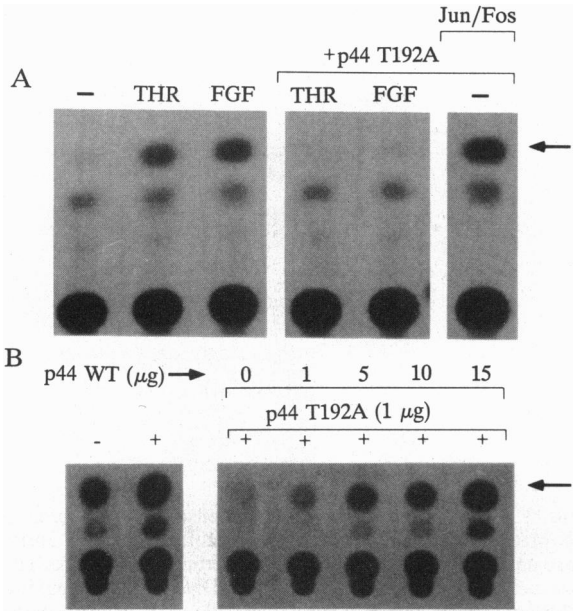

FIG. 4. Dominant-negative inhibition of growth factor-stimulated AP1 transcriptional activity. The −73/+63 collagen promoter–CAT reporter plasmid contains a single AP1 binding site responsible for growth factor induction of the collagenase gene (34). CCL39 cells (10^6) were transfected with 2 μg of the reporter plasmid together with the indicated expression vectors in a total of 30 μg of plasmid DNA (pUC19). (*A*) $p44^{mapk}$ T192A mutant inhibits growth factor stimulation of CAT activity. Cells were cotransfected with 5 μg of the empty expression vector pcDNA-neo (Invitrogen) or the same vector containing DNA coding for the $p44^{mapk}$ T192A mutant. Fourteen hours later, cells were serum-deprived for 24 hr and left untreated (−) or stimulated with 1 α-thrombin (THR; 1 unit/ml) or basic FGF (50 ng/ml) for an additional 12 hr. Growth factor-independent activation of AP1 activity as obtained by transfecting 1 μg of the expression vectors for c-Jun and c-Fos (lane Jun/Fos) was not affected by the expression of the $p44^{mapk}$ T192A mutant. This experiment is representative of three independent experiments. Arrows at right indicate the position of the acetylated chloramphenicol substrate. (*B*) Overexpression of $p44^{mapk}$ wild type reverses inhibition by the $p44^{mapk}$ T192A mutant. CCL39 cells were transfected as above in the presence of the indicated amounts of expression vector encoding $p44^{mapk}$. The total amount of expression vector was maintained constant to avoid competition for $p44^{mapk}$ T192A expression. Serum-starved cells (−) were stimulated by the addition of 10% fetal bovine serum (+) for 12 hr.

dation. It is of interest, however, that the expression of both MAP kinases, which share 85% identity at the protein level, was similarly suppressed by the expression of only one isoform of MAP kinase RNA antisense. At the highest concentration of antisense plasmid used, we obtained a 90% reduction in the expression of both MAP kinase isoforms after 3 days. Considering a protein half-life of about 24 hr for both MAP kinases (K. Seuwen, C. Kahan, and J.P., unpublished results), it is clear that $p44^{mapk}$ antisense expression induced the complete destruction of *de novo* synthesized MAP kinase RNA.

As for the second approach, the mechanism by which a $p44^{mapk}$ kinase-deficient mutant exerts a dominant negative effect remains to be elucidated. Clearly, marked overexpression of the mutant form is required to promote the inhibitory action and both MAP kinase isoforms are affected. At least two hypotheses can be put forward. Taking into account that MAP kinase functions as a monomer, the simplest hypothesis is that the $p44^{mapk}$ mutant form, expressed at high level, stops the growth factor signals by "titrating" the MAP kinase kinase, a common activator of both MAP kinases (15–19). Interestingly, the dominant negative effects can be reverted

Table 1. p44 MAP kinase-deficient mutants inhibit cell proliferation

cDNA construct	Colony no. after 1 week		
	6 μg	12 μg	18 μg
p44 Wild type	86	84	75
p44 Y194F	90	28	12
p44 T192A	50	22	0

PS120 cells (3×10^5 per 6-cm dish) grown in DMEM supplemented with 10% fetal bovine serum were cotransfected with 1 μg of pEAP vector together with 6, 12, or 18 μg of $p44^{mapk}$ constructs cloned in pcDNA-neo expression vector. Amounts of DNA were maintained constant (18 μg) with empty vector. Acid-load selection was applied at days 2, 6, and 8 after transfection and colonies were counted the next day, after staining. Successive acid-load selections are required to ascertain that all cells surviving are indeed expressing the Na^+/H^+ antiporter gene and consequently the transfected $p44^{mapk}$ alleles. A few nontransfected cells can escape the first acid load but are killed by the second selection. The third selection is usually not necessary, since >80% of the emerging clones that survived two acid loads will survive the third one. The data represent the number of colonies in a typical experiment that has been reproduced three times.

by coexpressing solely $p44^{mapk}$. Although this result suggests that $p42^{mapk}$ and $p44^{mapk}$ can substitute for each other, a knockout of each isoform will be necessary to definitively evaluate their specific targets and possibly overlapping functions. The second hypothesis is that overexpression of the $p44^{mapk}$ dead-kinase mutant interferes with nuclear translocation of both MAP kinases (ref. 37; P.L., C. Sardet, G.P., and J.P., unpublished results). This hypothesis is appealing because translocation of MAP kinase into the nucleus occurs within 15 min after addition of growth factor, it correlates with the mitogenic potential of the agonist, and phosphorylation of nuclear transcription factors is thought to be a critical functional feature of MAP kinases (38–40). To discriminate between these two hypotheses, one might use specific and precipitating anti-MAP kinase kinase antibodies to analyze the stability of the MAP kinase kinase/MAP kinase complexes with both wild-type and mutated forms of MAP kinase. With differential epitope tagging, one could investigate whether expression of the mutated $p44^{mapk}$ form interferes with nuclear translocation of endogenous MAPKs.

The main conclusion of this report is that growth factor activation of $p42^{mapk}$ and $p44^{mapk}$ is a prerequisite for fibroblast proliferation, a conclusion that reinforces the proposal that the persistent phase of MAP kinase activation is required for transition from G_0 to S phase (25, 26). The *ras* and *raf* protooncogenes, which encode two upstream actors of the MAP kinase cascade (20–22, 41, 42), have also been found to

Table 2. p44 MAP kinase antisense constructs inhibit cell proliferation

cDNA construct(s)	Colony no. after 1 week		
	5 μg	10 μg	15 μg
Sense p44mapk	55	50	59
Antisense p44, 240 bp	17	4	2
Antisense p44, 600 bp	11	4	2
Antisense p44, 850 bp	22	6	3
Antisense p44, 1750 bp	9	5	3
Antisense p44, 1750 bp, + sense p44mapk (10μg)	45	ND	ND

PS120 cells (3×10^5 per 6-cm dish) grown in DMEM supplemented with 10% fetal bovine serum were cotransfected with 1 μg of pEAP vector together with 5, 10, or 15 μg of antisense $p44^{mapk}$ constructs. Selection was identical to that described in the legend of Table 1. Antisense $p44^{mapk}$ constructs derived from the epitope-tagged $p44^{mapk}$ were subcloned into pECE vector. Three independent experiments performed with the 1750-bp antisense construct gave similar results. ND, not done.

be essential to propagate growth and differentiation signals (24, 43, 44). By extending this conclusion to downstream elements of the MAP kinase cascade, the model in which this cascade emerges as an obligatory and essential signaling path for growth factors and other extracellular signals becomes increasingly convincing. Here we have shown that MAP kinase suppression markedly inhibits cell growth. In preliminary experiments we found that microinjection of the $p44^{mapk}$ T192A mutant into G_0-arrested rat fibroblasts specifically prevented serum-induced DNA replication, suggesting that the cells are not able to pass the "restriction point" (C. Gauthier-Rouvière, G.P., P.L., and J.P., unpublished results; see ref. 45). "Start" and the "restriction point" are controlled by the activity of cdc2/G1 cyclins in yeast (46) and presumably by the activity of cdk2/cyclin E in human cells (47). It will therefore be of interest to analyze whether the persistent activation of MAP kinases, which appears to determine the $G_1 \rightarrow$ S-phase transition (25, 26), controls the activation of cdk2/G1 cyclin complexes. With the molecular tools developed in this study, it will be possible to answer this question and to investigate whether all or a subset of the multiple growth factor-activated targets (36) referred to as the "pleiotypic program" are controlled by the activation of MAP kinases. Here we have mainly emphasized the role of MAP kinases in the control of cell growth; however, we predict that MAP kinases are also essential in the initiation of differentiating programs. For example, we expect that nerve growth factor-induced differentiation of PC12 cells can be suppressed by specifically blocking MAP kinase activation. If this is the case, then this kinase cascade, conserved in yeast (48), should be considered as a "master signaling route" activating multiple cellular targets—in particular, transcription factors essential for growth and differentiation.

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