

Growth Factors Induce Nuclear Translocation of MAP Kinases (p42^{mapk} and p44^{mapk}) but not of Their Activator MAP Kinase Kinase (p45^{mapkk}) in Fibroblasts

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Abstract. Mitogen-activated protein kinases (p42^{mapk} and p44^{mapk}) are serine/threonine kinases that are activated rapidly in cells stimulated with various extracellular signals. This activation is mediated via MAP kinase kinase (p45^{mapkk}), a dual specificity kinase which phosphorylates two key regulatory threonine and tyrosine residues of MAP kinases. We reported previously that the persistent phase of MAP kinase activation is essential for mitogenically stimulated cells to pass the "restriction point" of the cell cycle. Here, using specific polyclonal antibodies and transfection of epitope-tagged recombinant MAP kinases we demonstrate that these signaling protein kinases undergo distinct spatio-temporal localization in growth factor-stimulated cells. In G0-arrested hamster fibroblasts the activator p45^{mapkk} and MAP kinases (p42^{mapk}, p44^{mapk}) are mainly cytoplasmic. Subsequent to mitogenic stimulation by serum or α -thrombin both MAP kinase isoforms translocate into the nucleus. This translocation is rapid

(seen in 15 min), persistent (at least during the entire G1 period up to 6 h), reversible (by removal of the mitogenic stimulus) and apparently 'coupled' to the mitogenic potential; it does not occur in response to nonmitogenic agents such as α -thrombin-receptor synthetic peptides and phorbol esters that fail to activate MAP kinases persistently. When p42^{mapk} and p44^{mapk} are expressed stably at high levels, they are found in the nucleus of resting cells; this nuclear localization is also apparent with kinase-deficient mutants (p44^{mapk} T192A or Y194F). In marked contrast the p45^{mapkk} activator remains cytoplasmic even during prolonged growth factor stimulation and even after high expression levels achieved by transfection. We propose that the rapid and persistent nuclear transfer of p42^{mapk} and p44^{mapk} during the entire G0-G1 period is crucial for the function of these kinases in mediating the growth response.

THE sequence of events which link cell surface receptor-mediated signals to expression of a specific pattern of genes has received considerable attention in recent years. Although many gaps remain to be filled, the remarkable degree of homology that is emerging between yeast and mammalian signaling pathways should help in elucidating all sequential events. In particular, the linear cascade of yeast protein kinases: byr2 (ste11)→byr1 (ste7)→spk1 (FUS3/KSS1), linking pheromone receptor to gene expression (Nielsen, 1993) is amazingly well conserved in mammalian cells. The corresponding mammalian "signaling module" is mitogen-activated protein (MAP)¹ kinase kinase kinase (MAPKKK)→MAP kinase kinase (MAPKK)→MAP kinases (MAPK) (Lange-Carter et al., 1993; Neiman et al., 1993). MAP kinases, also described as extracellular-regulated kinases (ERK), belong to a family of serine/threonine protein kinases (Sturgill and Wu, 1991; Boulton et al., 1991;

Pelech et al., 1992) that have been shown to participate in signaling pathways initiated by mitogens or differentiating agents. In mammalian cells there are at least two highly homologous MAP kinases: p42^{mapk} or ERK2 and p44^{mapk} or ERK1 (Boulton et al., 1991). They share the same substrates in vitro (Sturgill et al., 1988; Pulverer et al., 1991; Northwood et al., 1991; Alvarez et al., 1991; Gille et al., 1992) and the same time course of activation (Meloche et al., 1992a). They are expressed ubiquitously (Boulton et al., 1991) and closely related to the yeast protein kinases involved in pheromone-induced mating: SLT2 (Torres et al., 1991), KSS1 (Courchesne et al., 1989), and FUS3 (Elion et al., 1990) in budding yeast and spk1 (Toda et al., 1991) in fission yeast. This homology of sequence also reflects a similarity in function since spk1 defect of *S. pombe* can be complemented by expressing either rat p42^{mapk} (Neiman et al., 1993) or hamster p44^{mapk} (Nielsen, O., and J. Pouyssegur, unpublished results).

In fibroblasts, MAP kinases undergo a rapid and biphasic activation in response to all mitogens (Meloche et al.,

1. Abbreviations used in this paper: aa, amino acid; MAP, mitogen-activated protein.

1992a) that parallels their direct phosphorylation on both threonine and tyrosine residues (Ray and Sturgill, 1988; Anderson et al., 1990) by a dual specificity kinase of 45 kD termed the MAP kinase activator or MAP kinase kinase (p45^{mapkk}) (Ahn et al., 1990; Nakielny et al., 1992; Seger et al., 1992; Roberts, 1993; Kosako et al., 1993). In turn, p45^{mapkk} is activated by phosphorylation of serine/threonine residues (Kosako et al., 1992) and corresponds to the yeast homologs *byr1/ste7*. One potential upstream activator of p45^{mapkk} is the proto-oncogene *c-raf*, since immunoprecipitated *v-raf* and activated *c-raf* can re-activate dephosphorylated p45^{mapkk} (Kyriakis et al., 1992; Dent et al., 1992; Howe et al., 1992). Most interesting, however, is the recent discovery of another mammalian activator of MAP kinase kinase; it is a serine/threonine kinase of 78 kD, also regulated by phosphorylation, and found to be analogous to the yeast *byr2/stell1* (Lange-Carter et al., 1993).

We have shown previously that MAP kinases are essential for G0 to S-phase transition in fibroblasts. First, we showed that the growth factor-induced second and persistent phase of MAP kinase activation correlates with the mitogenic potential of the stimulus (Meloche et al., 1992a) and second that inhibition of both MAP kinases by expression of either a p44^{mapk} antisense cDNA or dominant-negative allele suppresses cell growth (Pagès et al., 1993). Here, using indirect immunofluorescence microscopy, we demonstrate that both p42^{mapk} and p44^{mapk} rapidly and persistently translocate to the nucleus in response to strong mitogens whereas their common activator, p45^{mapkk}, remains cytoplasmic. This finding supports the notion that these kinases play a critical role in relaying surface receptor-mediated signals to the nucleus.

Materials and Methods

Materials

Highly purified human α -thrombin and basic FGF were generous gifts of Drs. J. W. Fenton II (New York State Department of Health, Albany, NY) and D. Gospodarowicz (University of California Medical Center, San Francisco, CA), respectively. BSA (fraction V) was obtained from Sigma Immunochemicals (St Louis, MO). Synthetic peptides C17p44 (CIFQETARFQPGAPEAP) corresponding to the rat p44^{mapk} 16 carboxyl-terminal amino acid (aa) and the synthetic peptide C15p42 (CIFEETARFQPGYRS) corresponding to the rat p42^{mapk} 14 carboxyl-terminal aa were both synthesized with a Cysteine at the amino terminus by Dr. G. Drapeau (Université Laval, Québec, Canada). The rabbit polyclonal antiserum 837 (Boulton et al., 1991) was kindly provided by Dr. M. Cobb (University of Texas Southwestern Graduate school of Biomedical Sciences, Dallas, TX) which was raised against the synthetic peptide (IFQETARFQPGAPEAP) corresponding to the rat p44^{mapk} 16 carboxyl-terminal aa. Fluorescein-conjugated anti-rabbit and anti-mouse IgG from sheep were obtained from Amersham (UK). A synthetic peptide N16p45 (PKKKPTPIQLNPNPEY) corresponding to the *Xenopus laevis* p45^{mapkk} 16 amino-terminal aa and synthetic hamster α -thrombin receptor peptide (7-mer = SFFLRNP) were synthesized by Neosystem SA (Strasbourg, France). mAb P4D5 which recognizes the epitope YTDIEMNRLGK (VSVG; Kreis, 1986) was kindly provided by Dr. B. Goud (Institut Pasteur, Paris, France). A mouse p42^{mapk} cDNA clone (pBA4) was kindly provided by Prof. M. Weber (University of Virginia, Charlottesville, VA).

Cell Line and Cell Culture

The Chinese hamster lung fibroblast cell line CCL39 (ATCC) and its derivative TR4-44* overexpressing an epitope-tagged p44^{mapk} (Meloche et al., 1992a,b) were cultured in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 7.5% FCS and antibiotics (50 U/ml penicillin and 50 μ g/ml

streptomycin) at 37°C in an atmosphere of 95% air, 5% CO₂. Quiescent cells (G0-arrested) were obtained by incubating confluent cultures in serum-free medium for 24–48 h. Cells were then treated with various agents as described in Results and figure legends.

Western Blot Analysis

Confluent cells were lysed in Triton X-100 lysis buffer (0.2% Triton X-100, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM PMSF, 1 mM iodoacetamide, 1 mM *o*-phenanthroline). 25 μ g of detergent-extracted proteins were separated by SDS-PAGE on 10% (wt/vol) polyacrylamide gel (29:1), electrophoretically transferred to Hybond-C membranes (Amersham, UK) and immuno-probed as described previously (Meloche et al., 1992a).

Immunoprecipitation of p44^{mapk} and MBP Kinase Assay

The kinase activity was measured in presence of 25 μ M of nonradioactive ATP and 3 μ Ci of γ -³²P-ATP per assay for 15 min at 30°C, essentially as described previously by Meloche et al. (1992a) except that 1 μ l of 837p antiserum was added per assay (one well of a 12-well plate). Each assay was normalized per 1 μ g of protein present in cell lysates.

Indirect Immunofluorescence Microscopy

G0-arrested or serum-stimulated CCL39 cells and transfectants were washed with PBS and fixed at –20°C for 10 min with methanol/acetone (3:7, vol/vol). After a 10-min rehydration at 25°C in PBS containing 3% BSA (PBS/BSA), fixed cells were then incubated with the primary antibody for 60 min at 25°C. Cells were then washed five times with PBS and incubated in PBS/BSA for 60 min at 25°C with fluorescein-conjugated secondary antibody (anti-mouse or anti-rabbit, 1:50). Finally, cells were washed five times with PBS and examined under epifluorescent illumination with excitation-emission filters for fluorescein. To control for specificity: (a) the same results were obtained when cells were fixed with 1% paraformaldehyde in methanol at –20°C for 15 min or with 4% paraformaldehyde in PBS at 20°C for 15 min followed by permeabilization with 0.2% Triton X-100 in PBS for 5 min at 20°C (data not shown); (b) incubation with secondary antibody alone did not reveal any significant fluorescent signal (data not shown).

Immunopurification of Antibody MKK16

Antiserum MKK16 was obtained from a rabbit injected with the N16p45 peptide coupled to KLH (keyhole limpet hemocyanin). The antiserum MKK16 was either used directly (1:2,000) or immunopurified as follows: 100 μ l of pre-immune and MKK16 antisera were incubated overnight at 4°C with 25 μ g of peptide N16p45 coupled to KLH, bound to Hybond C-super membranes (Amersham, UK). After five washes with TN buffer (50 mM TrisCl, 150 mM NaCl, pH 8.0), antibody bound to the membrane was eluted for 30 min at 20°C with 100 μ l of the “ImmunoPure gentle Ag/Ab elution buffer” from Pierce (Rockford, IL). This solution was then dialyzed on Millipore filters (type VSWP02500) against TN buffer for 2 h at 4°C. Immunofluorescence microscopy was then performed as described above, except that PBS was replaced by TN buffer. Similar results were obtained with antibody MKK16-b, obtained from another rabbit (data not shown).

DNA Constructs and Expression Vectors

EcoRV restriction sites were introduced in the terminal stop codons of both p44^{mapk} (Meloche et al., 1992a) and p42^{mapk} (L'Allemain et al., 1992a), by the method of Kunkel (Bio Rad mutagenizing kit; BioRad Labs, Hercules, CA) to add the sequence WEGPPGPYTDIEMNRLGK (*hinge*-VSVG epitope; Kreis, 1986). These epitope-tagged MAP kinases were subcloned in the expression vector pcDNA-neo. The amino acid substitutions presented in the results section at positions 192 and 194 were introduced in the Chinese hamster p44^{mapk} cDNA by the method of Kunkel. p44^{mapk}-VSVG Δ 239 was obtained by creating an EcoRV site at the residue 239 and adding the P4D5 epitope recognition sequence at this position, thus deleting 129 aa's out of the 369 aa's of Chinese hamster p44^{mapk} (truncation of 30%).

Results

Specificity of the p44^{mapk} Antiserum 837

Rabbit antiserum 837 (Boulton et al., 1991) was raised

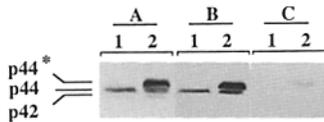


Figure 1. Western blot demonstrating specificity of antiserum 837 for p44^{mapk}. Antiserum 837 was used to detect p44^{mapk} (p44) in 25 μg of detergent-extracted proteins

from Chinese hamster lung fibroblasts CCL39 (lanes 1) and from a CCL39 transfectant (TR4-44*) overexpressing an epitope-tagged p44^{mapk} (p44*) (lanes 2). The antiserum 837 was used either directly (1:1,500) (A), or after blocking with p42-antigenic peptide C15p42 (150 μg/ml with 180 min preincubation) (B), or after blocking with C15p42 (150 μg/ml) and the p44 antigenic peptide C17p44 (450 μg/ml) (C).

against a 17-aa synthetic peptide corresponding to the predicted carboxy terminus of p44^{mapk}. Its specificity was tested by immunoblotting (Fig. 1 A) total cell lysates of both CCL39 fibroblasts and of TR4-44* cells, a CCL39 transfectant overexpressing an epitope-tagged p44 MAP kinase (p44*^{mapk}) (Meloche et al., 1992b). 837 antiserum recognizes predominantly p44^{mapk} (44 kD) and p44*^{mapk} but also weakly p42^{mapk} (Fig. 1 A). However, this antibody became monospecific for p44^{mapk} after preincubation with a 15-aa synthetic peptide corresponding to the carboxy terminus of the p42 MAP kinase (antiserum 837p, Fig. 1 B). This procedure was used for the immunolocalization of p44^{mapk}. The specific recognition of p44^{mapk} and p44*^{mapk} was blocked by pre-incubation with the p44 antigenic peptide (Fig. 1 C).

Immunolocalization of MAP Kinases in Resting and Serum-stimulated CCL39 Fibroblasts

Immunofluorescence microscopy was performed with 837p antiserum on G0-arrested and serum-stimulated CCL39 cells. To ensure that the staining observed was specific, several different fixation-permeabilization procedures were used for sample preparation (methanol/acetone, 4% paraformaldehyde, methanol/paraformaldehyde), and in each case it was ensured that the signal obtained could be extinguished by pre-incubation of diluted antisera with the corresponding antigenic p44 peptide or both the p44 and p42 antigenic peptides as shown in Fig. 2 F. As illustrated in Fig. 2, the observed staining for p44^{mapk} shows first that in G0-arrested CCL39 cells, p44^{mapk}-immunolabeling was cytoplasmic and a diffuse staining suggested no association with any organized cellular structure (Fig. 2 A). Second, as judged by immunofluorescence microscopy, p44^{mapk} underwent a complete redistribution in serum-stimulated cells, starting to move to the nucleus as early as 15 min (Fig. 2 B) and becoming predominantly nuclear 1–3 h after serum-stimulation (Fig. 2, C and D). However, this nuclear translocation was transient, p44^{mapk} starting to return to the cytoplasm 6 h after serum stimulation (data not shown), to be again totally cytoplasmic between 6 and 9 h after serum stimulation (data not shown). This timing suggests that p44^{mapk} nuclear translocation is a G0 to S-phase transition-specific event. The nuclear efflux of p44^{mapk} can also be triggered by removal of serum 1 h after stimulation. Exit of p44^{mapk} from the nucleus is partial 30 min after serum removal (compare Fig. 2, C and E) and complete at 1 h (data not shown). This demonstrates that continuous serum stimulation of cells is necessary to maintain p44^{mapk} in the nucleus, at least during the initial period of stimulation.

MAP Kinase Remains Active during the Period of Nuclear Localization

As illustrated in Fig. 3, MAP kinase activity immunoprecipitated by 837p antiserum, was measured by phosphorylation of MBP in serum-starved and serum-stimulated CCL39 fibroblasts (by 10% FCS). As previously described for CCL39 cells (Meloche et al., 1992a; Kahan et al., 1992) the activation of endogenous p44^{mapk} is biphasic in response to potent mitogens such as 10% serum or α-thrombin; a rapid activation occurring between 5 and 10 min after agonist addition is followed by a second broad wave of activation lasting at least 3 h. We show here that this second peak of activity decreased thereafter continuously from 3 to 9 h after serum stimulation. However, it never returned to the basal level observed in G0-arrested cells (four out of five trials) indicating that MAP kinase activity is still elevated over basal levels at times when nuclear localization of this kinase is still observed (Figs. 2 and 3). Thus, after translocation to the nucleus, part of the active MAP kinase pool could phosphorylate several of its in vitro substrates such as the nuclear proteins c-jun (Pulverer et al., 1991; Baker et al., 1992), c-myc (Alvarez et al., 1991) and p62^{TCF} (Gille et al., 1992). Note that removal of serum growth factor induced a rapid deactivation of MAP kinase concomitant with its disappearance from the nucleus.

The Potency of Growth Promoting Agents to Induce MAP Kinase Nuclear Translocation Correlates with Their Relative Potency to Promote S-phase Entry

We have shown in this laboratory that the sustained second phase of p44^{mapk} kinase activation correlates with mitogenicity since it is observed in cells stimulated with mitogenic agents such as α-thrombin or FGF but not after stimulation by non-mitogenic agents such as carbachol (Pouyssegur and Seuwen, 1992; Kahan et al., 1992), phorbol esters or α-thrombin receptor peptide (Vouret et al., 1993). Fig. 4 shows that a similar correlation was observed for the p44^{mapk} nuclear translocation; potent mitogens induced nuclear translocation whereas weak or co-mitogens did not. For example, treatment of G0-arrested cells CCL39 cells for 3 h with non-mitogenic stimuli, such as phorbol esters (data not shown) or α-thrombin receptor peptide (Fig. 4 B) failed to induce p44^{mapk} nuclear translocation. In contrast, growth factors such as α-thrombin (Fig. 4 A) or FGF (data not shown) were able to promote G0/G1 p44^{mapk} nuclear translocation. Although these mitogens were less potent than 10% serum in their ability to trigger p44^{mapk} nuclear translocation when added individually, they became equivalent to serum when added together (α-thrombin/FGF, Fig. 4 C). Interestingly, the intensity and percentage of nuclei harboring p44^{mapk} immunolabeling seemed to correlate well with the relative potency of these mitogens to re-initiate DNA synthesis (Fig. 4) (Vouret-Craviari et al., 1992).

Immunolocalization of p42^{mapk} Isoform in CCL39 Fibroblasts

Our attempts to obtain antisera that exclusively recognised p42^{mapk} in immunoblotting were unsuccessful; all antisera tested presented some reactivity against p44^{mapk}.

Thus, we resorted to transfecting epitope-tagged isoforms to study their specific subcellular localization. We added 18

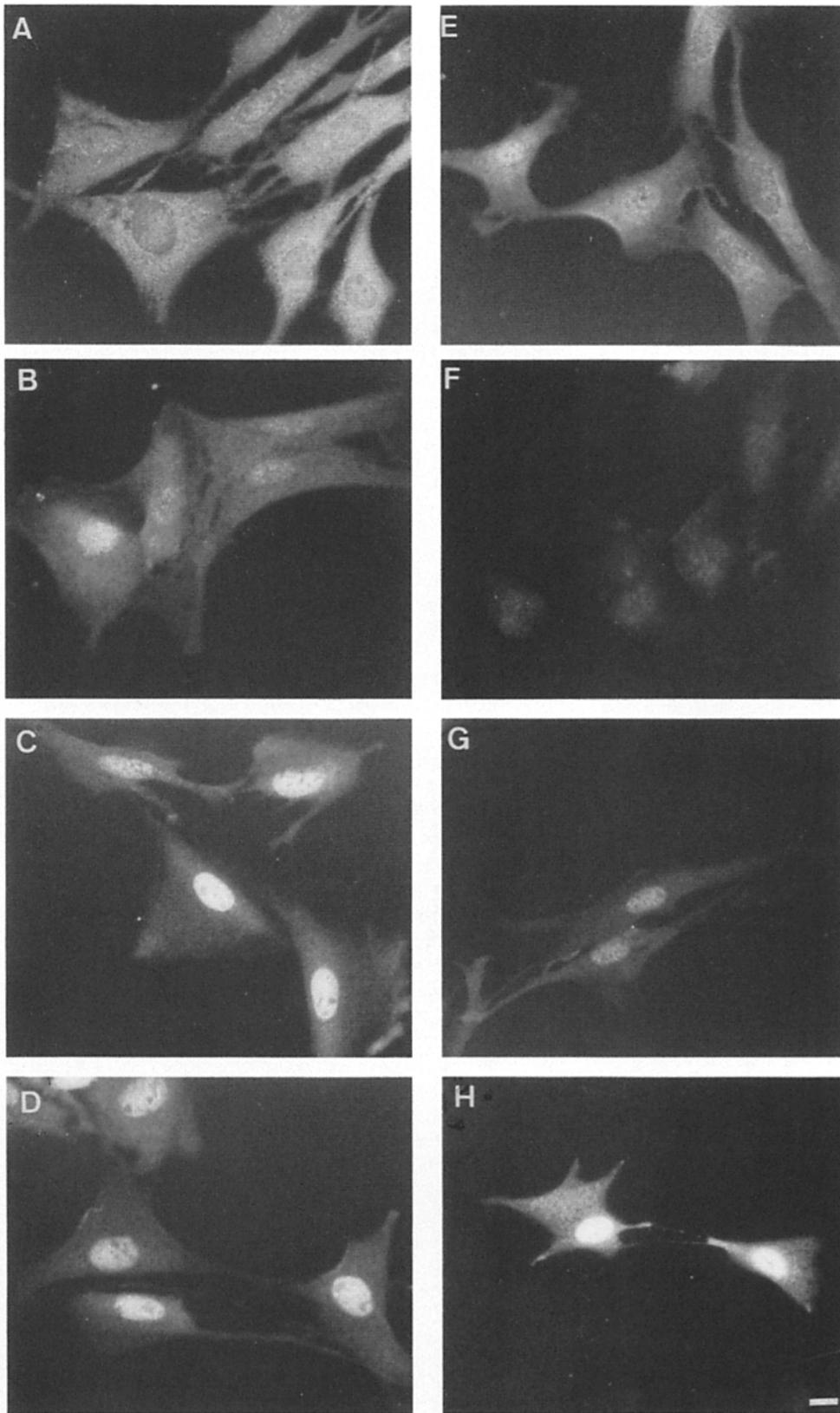


Figure 2. Indirect immunofluorescence microscopy showing p44^{mapk} nuclear translocation after serum stimulation of G0-arrested cells. CCL39 fibroblasts were incubated in serum-free medium for 2 d (G0-arrested) (A) and then were stimulated by the addition of 10% FCS for 15 (B), 60 (C) or 180 (D and G) min. Similarly, G0-arrested TR4-44* cells were stimulated for 180 min with 10% serum (H). In E, cells were stimulated with 10% FCS for 60 min, washed thoroughly and incubated in serum-free medium for 30 min before fixation. Cell fixation and immunofluorescence labeling procedures were performed as described in Materials and Methods. The first antibody was the 837 antiserum (1:400) blocked with C15p42 (150 μg/ml) (A-E) or blocked simultaneously with peptides C15p42 (150 μg/ml) and C17p44 (450 μg/ml) (F). The difference in p44 specific fluorescence labeling between CCL39 cells and TR4-44* cells was demonstrated using a higher dilution (1:1,000) of the 837 antiserum (compare panels G and H). Cells were obtained with a Nikon Diaphot microscope, objectif fluor 40, 1.3 oil. Bar, 10 μm.

aa's corresponding to the epitope recognized by the mAb VSVG and a hinge region (Kreis, 1986) at the carboxy terminus of the two MAP kinases isoforms; these proteins are termed p42^{mapk-VSVG} and p44^{mapk-VSVG}. As shown in Fig. 5,

serum-stimulated CCL39 cells expressing either p44^{mapk-VSVG} (Fig. 5 A) or p42^{mapk-VSVG} (Fig. 5 B) showed specific immunolabeling located mostly in the nucleus with the exclusion of nucleoli (observed with the VSVG epitope-specific

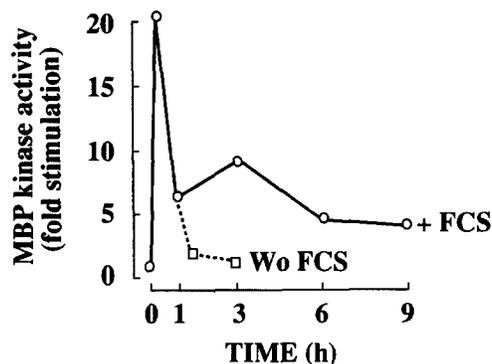


Figure 3. Time course of MBP kinase activation in response to 10% FCS. MBP kinase activity was measured and normalized as described in Materials and Methods. Confluent CCL39 cells (12-well plates) were serum deprived for 24 h, stimulated with 10% FCS and then harvested either 1, 3, 6, or 9 h after stimulation (+FCS). Alternatively, cells were stimulated for 1 h, and then washed three times with serum-free medium and harvested 30 min or 3 h later (Wo FCS). The data presented are representative of five experiments which gave similar results.

mAb P4D5, see Materials and Methods). In both cases (Fig. 5, *A* and *B*) nonexpressing cells showed a weak background staining notably excluded from the nucleus. The VSVG-specific staining was observed with cells fixed either with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 or for cells fixed with methanol/acetone (30:70%) at -20°C (Materials and Methods). Furthermore, no immunofluorescent signal was detected in cells transfected with p44^{mapk}-(Δ VSVG) harboring a truncated VSVG epitope (data not shown). From these experiments, we conclude that transfected proteins p42^{mapk}-VSVG and p44^{mapk}-VSVG are located in the nucleus of serum-stimulated CCL39 cells.

It is important to note that the expression level of each full-length transfected isoform (WT-p42^{mapk}, p44^{mapk}, p42^{mapk}-VSVG, and p44^{mapk}-VSVG) altered their subcellular localization in serum-starved cells, a high level of expression induced a partial nuclear localization in serum-starved cells. Cytoplasmic MAP kinase localization in serum-deprived cells and nuclear translocation to the nucleus after serum stimulation was observed only in transfectants expressing low levels of this protein (data not shown).

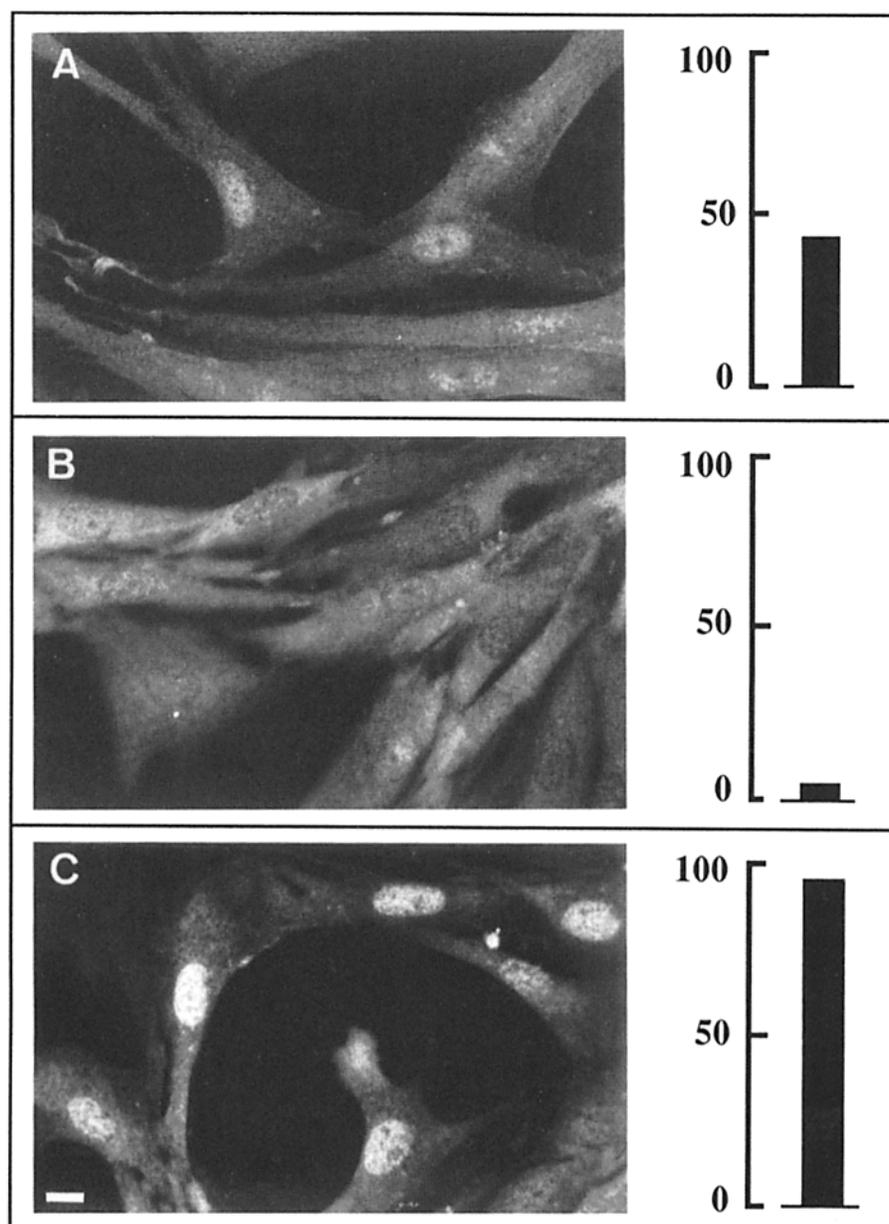


Figure 4. Immunofluorescence labeling of p44^{mapk} showing a correlation between mitogenicity and nuclear translocation. G0-arrested CCL39 fibroblasts were stimulated for 180 min with 10 U/ml α -thrombin (*A*), 100 $\mu\text{g/ml}$ of 7-mer synthetic α -thrombin receptor peptide (*B*), or simultaneously with 10 U/ml α -thrombin and 25 ng/ml FGF (*C*). Immunofluorescence labeling procedures were performed as described in Materials and Methods with antiserum 837p (1:400). Solid black bars represent the reinitiation of DNA synthesis expressed as the percent of the maximal [^3H]thymidine incorporation obtained with 10% FCS measured after 24 h as described previously (Vouret-Craviari et al., 1992). Bar, 10 μm .

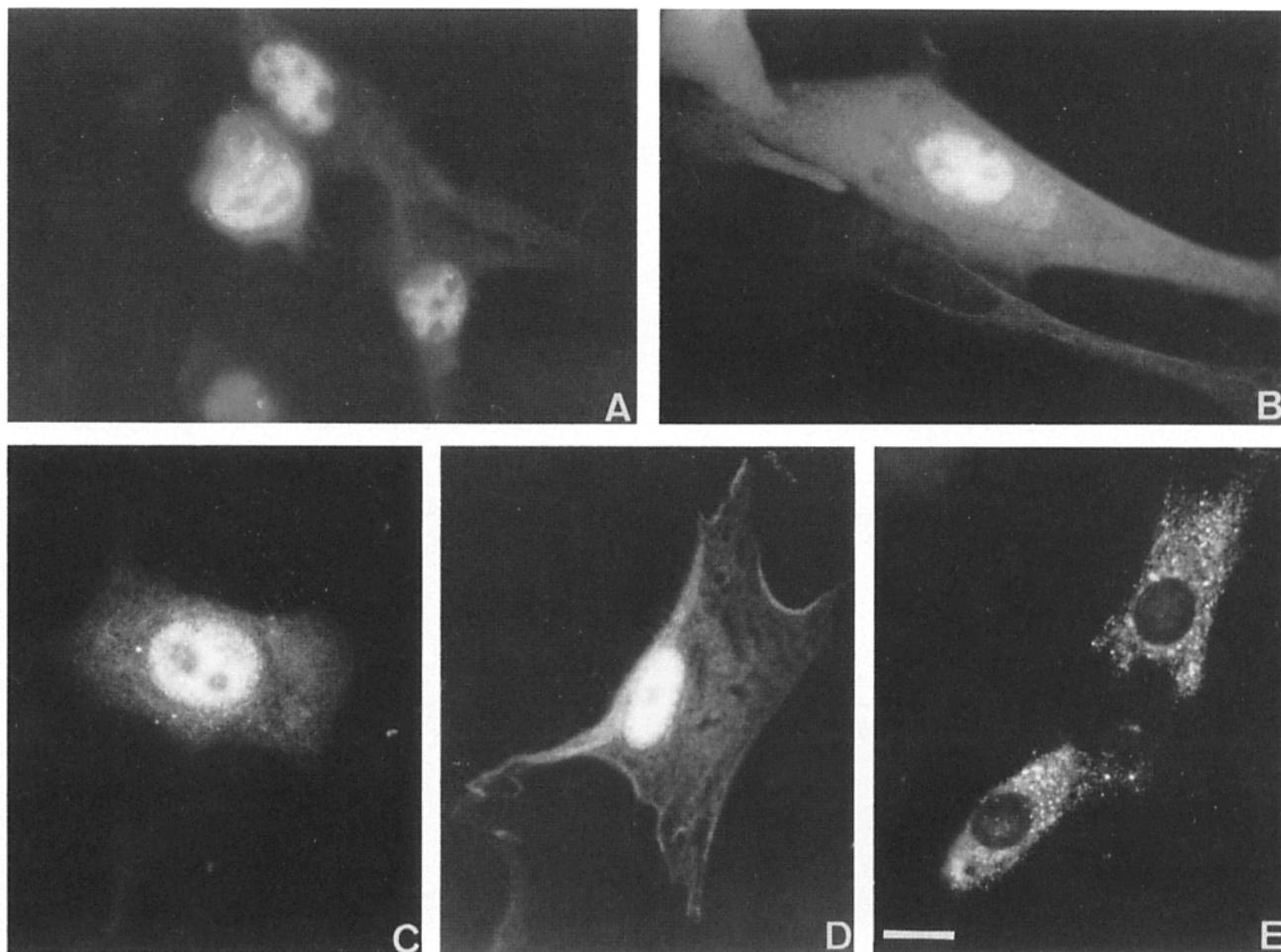


Figure 5. Indirect immunofluorescence microscopy of CCL39 cells expressing epitope-tagged MAP kinases. Immunofluorescence labeling procedures of epitope-tagged MAP kinases were performed as described in Materials and Methods with the P5D4 mAb as primary antibody (1:500) and anti-mouse IgG coupled to fluorescein as secondary antibody (1:50). Serum-stimulated CCL39 cells expressing the following epitope-tagged MAP kinases constructs are presented: (A) p44^{mapk-VSVG}, (B) p42^{mapk-VSVG}, (C) p44^{mapk-VSVG} T192A, (D) p44^{mapk-VSVG} T192E-Y194E, and (E) p44^{mapk-VSVG-Δ239}. Cells were observed with a Nikon Diaphot microscope, objective fluor 100, 1.3 oil. Bar, 10 μ m.

We conclude that both p42^{mapk-VSVG} and p44^{mapk-VSVG} translocate to the nucleus after growth factor stimulation, however when highly expressed their location is both cytoplasmic and nuclear in serum-starved cells.

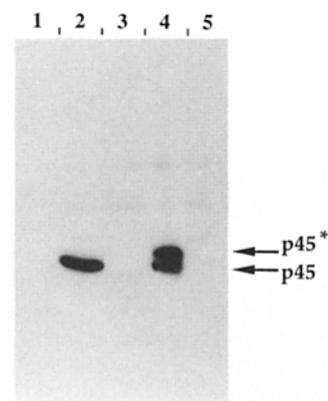


Figure 6. Western blot demonstrating specificity of MKK16 antiserum for p45^{mapk}. MKK16 antiserum was used to detect p45^{mapk} (p45) in detergent-extracted proteins from CCL39 fibroblasts (lanes 1, 2, and 3) and of the CCL39 transfectant (CCL39-p45) overexpressing an epitope-tagged p45^{mapk} (p45*) (lanes 4 and 5). Each lane contained 25 μ g of detergent-extracted proteins. The Western blots were probed with pre-immune serum (lane 1), antiserum MKK16 at 1:2,000 dilution (lanes 2 and 4), and MKK16 (1:2,000) blocked with 10 μ g/ml MKK16-antigenic peptide N16p45 preincubated for 30 min at 37°C (lanes 3 and 5).

MAP Kinase Nuclear Localization Occurs with Kinase-dead Mutants

MAP kinase activation results from phosphorylation of T192 and Y194 residues by the activator p45^{mapk}. We and others have shown that mutations of either of these phosphorylation sites totally inactivate MAP kinases (Posada and Cooper, 1992; L'Allemain et al., 1992b; Pagès et al., 1993). We decided to test whether the activation of MAP kinases by phosphorylation was necessary for nuclear translocation. Thus, by site-directed mutagenesis of p44^{mapk-VSVG} we removed the key phosphorylation sites (T192A, Y194F) or substituted them by negatively charged residues (T192E, Y194E). Different combinations of these mutations were tested in stable transfected clones. As shown in Fig. 5 the T192A (Fig. 5 C) and T192E-Y194E (Fig. 5 D) mutations that abolish MAP kinase activity did not alter the nuclear translocation. Subcellular localization of these mutants, including Y194F, T194E, and Y194E (data not shown) was indistinguishable from that of transfected wild type p44^{mapk-VSVG} (Fig. 5 A). In addition, serum-induced nuclear translocation of these mutants can be detected in cells with low expression levels.

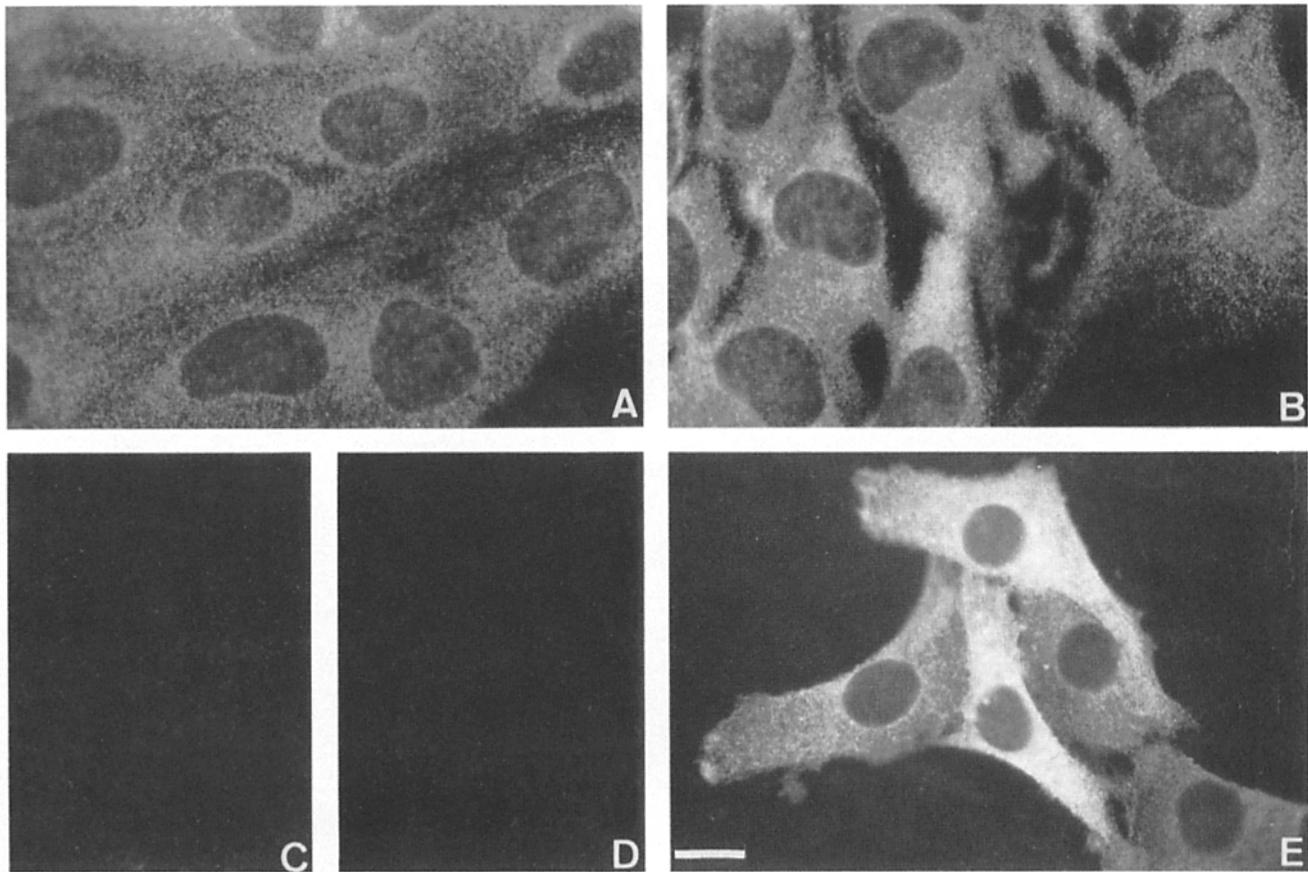


Figure 7. Indirect immunofluorescence microscopy of the MAP kinase activator, p45^{mapkk}. Immunofluorescence labeling procedures of p45^{mapkk} were performed as described in Materials and Methods with CCL39 cells (A–D) and p45^{mapkk} transfected-CCL39 cells (E). G0-arrested CCL39 fibroblasts (A) were stimulated with 10% FCS (B–E). The primary antibodies were immunopurified MKK16 antibody (1:50) (A and B); immunopurified MKK16 (1:50) blocked for 30 min at 37°C with the antigenic peptide N16p45 (10 µg/ml) (C); immunopurified pre-immune MKK antibody (1:50) (D); and antibody MKK16 (1:2,000) (E). Bar, 10 µm.

We conclude that the transfer and the retention of MAP kinases in the nucleus is independent of the state of MAP kinase activity.

Characterization of p45^{mapkk} Antibody

As shown on Fig. 6, lane 3, rabbit-antiserum MKK16 raised against the NH₂-terminal peptide of *Xenopus* MAP kinase kinase (peptide N16p45, see Material and Methods), specifically recognized a single band with an apparent mass of ~45 kD on immunoblots prepared from cytosolic extracts of CCL39 cells. Pre-immune antiserum and MKK16-antiserum blocked with the antigenic peptide N16p45 failed to display any specific labeling (Fig. 6, lanes 1 and 2, respectively). In addition, MKK16 antiserum recognized recombinant p45^{mapkk*} stably transfected in CCL39 cells (Fig. 6, lane 4). The specific recognition of p45^{mapkk*} was also blocked by pre-incubation of MKK16-antiserum with the antigenic peptide N16p45 (Fig. 6, lane 5). p45^{mapkk*} displayed a retarded migration in SDS-PAGE due to the addition of the HA1 epitope at the amino terminus of the Chinese hamster p45^{mapkk}-cDNA (Pagès, G., A. Brunet, G. L'Allemain, P. Lenormand, and J. Pouyssegur, manuscript in preparation). Another rabbit antiserum (MKK16-b) raised independently against the same peptide N16p45, displayed similar specificity and immunoreactivity (data not shown).

Furthermore, these antisera immunoprecipitated a protein kinase from CCL39 cytosolic extracts able to tyrosine-phosphorylate and re-activate recombinant inactive p42^{mapk}. These data confirm the immunoreactivity of these antisera with the mammalian MAP kinase activator p45^{mapkk} (Pagès, G., A. Brunet, G. L'Allemain, P. Lenormand, and J. Pouyssegur, manuscript in preparation).

Cellular Immunolocalization of the MAP Kinase Activator p45^{mapkk}

Using indirect-immunofluorescence microscopy with immunopurified MKK16 antiserum, we localized p45^{mapkk} in the cytoplasm of serum-starved CCL39 cells with no apparent association with organized cellular structure (Fig. 7 A). After 3 h of serum stimulation, p45^{mapkk} was still cytoplasmic (Fig. 7 B). In fact p45^{mapkk} remained in the cytoplasm of exponentially growing CCL39 cells (data not shown) and even when it was overexpressed (Fig. 7 E). This cytoplasmic localization was also observed in other fibroblastic and epithelial cell lines (Swiss 3T3, COS 7, MDCK, data not shown). To ensure that the observed staining was specific, two fixation-permeabilization procedures were used for sample preparation: methanol/acetone (30:70%) and 4% paraformaldehyde/0.2% Triton X-100, and in each case we demonstrated that the signal could be extinguished by pre-

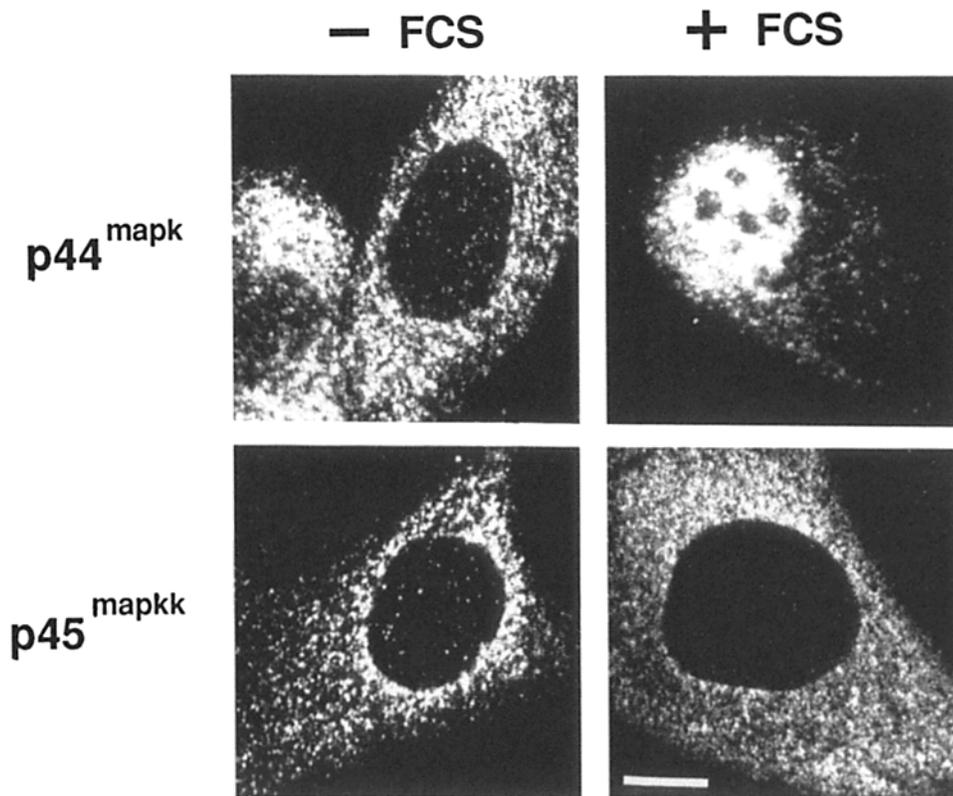


Figure 8. Confocal laser scanning microscopy of p45^{mapkk} and p44^{mapk}. Immunofluorescence labeling procedures were performed as described in Materials and Methods with CCL39 cells, serum-starved for 24 h (-FCS) or the same cells after stimulation with 20% FCS for 3 h (+FCS). The antisera 837p (1:500) and MKK16 (1:1,000) were used for immunodetection of p44^{mapk} and p45^{mapkk}, respectively. Optical sections in the center of nuclei were performed at 2.1 μm from the bottom of the cells using a Leica confocal microscope. Bar, 5 μm .

incubation of diluted antisera with the antigenic peptide (Fig. 7 C) and strongly increased after transfection of p45^{mapkk} cDNA (Fig. 7 E). Immunopurified pre-immune sera from the same rabbit did not display any specific immunoreactivity (Fig. 7 D).

Confocal laser scanning microscopy was performed to confirm the absence of p45^{mapkk} in the nucleus. Optical sections passing through the center of nuclei were obtained with CCL39 cells labeled either with antisera MKK16 antisera to detect p45^{mapkk}, or antisera 837p to detect p44^{mapk}. These data, presented in Fig. 8 demonstrate the total absence of p45^{mapkk} in the nucleus of both serum-stimulated and non-stimulated CCL39 cells. This situation sharply contrasts with the redistribution of p44^{mapk} in the same cells upon stimulation with serum.

We conclude that although both MAP kinase isoforms translocate to the nucleus after growth factor stimulation, their upstream activator p45^{mapkk} remains strictly cytoplasmic during the cell cycle.

Discussion

In this study, we were able to examine specifically the subcellular localization of endogenous p44^{mapk}. Clearly, endogenous p44^{mapk} translocated to the nucleus after growth factor stimulation of G0-arrested CCL39 cells. The time course of p44^{mapk} translocation to the nucleus indicated that p44^{mapk} was present in the nucleus soon after stimulation (at least 15 min), and remained nuclear up to 6 h after growth factor stimulation. This process parallels the long lasting phase of MAP kinase activation. Interestingly, growth factor removal rapidly reverses the process of p44^{mapk} nuclearization and abolishes its activation. It is thus very likely that p44^{mapk} is

indeed active when it appears in the nucleus after growth factor stimulation. Using an epitope-tagging strategy, we were able to demonstrate that transfected p42^{mapk} also is nuclear upon growth factor stimulation. The same conclusion was reached when p44^{mapk} and p42^{mapk} were analyzed in rat mesangial cells stimulated with either serum or endothelin (Wang, Y., J. Pouyssegur, and M. Dunn, unpublished results). Hence, we must conclude that both p42^{mapk} and p44^{mapk} which share high homology, a common activator and identical in vitro substrate, both translocate to the nucleus. This implies that some of the known in vitro substrates of MAP kinases, such as the nuclear transcription factors *c-jun* (Pulverer et al., 1991; Baker et al., 1992), *c-myc* (Alvarez et al., 1991), and p62^{TCF} (Gille et al., 1992) can also be phosphorylated in vivo by MAP kinases. Our findings confirm and extend the previous report of Chen et al. (1992) on nuclear translocation of both MAP kinase isoforms in the nucleus of HeLa cells. These authors did not possess an isoform-specific antibody, thus they were unable to distinguish between the two ubiquitously expressed isoforms p42^{mapk} and p44^{mapk}. Our present study also confirms and extends the observations of Sanghera et al. (1992) and Seth et al. (1992) showing respectively that avian homolog of MAP kinase is nuclearly located and that transfected p42^{mapk} into cos-7 cells not only is nuclear but also able to phosphorylate Ser62 of the transactivation domain of *c-myc*.

MAP kinases are activated rapidly by a wide variety of agents which include mitogens, neurotransmitters, differentiating agents, phorbol esters, heat shock, etc. (Pelech et al., 1992). Here we have demonstrated that among the agonists that are capable of inducing a mitogenic response in CCL39 cells, only the strong mitogens such as α -thrombin, FGF, or serum that are known to elicit a persistent MAP kinase acti-

vation (Meloche et al., 1992b; Kahan et al., 1992), stimulate efficiently p44^{mapk} nuclear translocation. Synergistic mitogens like α -thrombin/FGF for DNA replication also synergize for the number of nuclei scored positive for p44^{mapk} immunostaining. Chen et al. (1992) have shown that phorbol esters induce nuclear translocation of MAP kinases in the HeLa tumor cell line. In CCL39 cells, however, phorbol esters that activate only the first and transient peak of MAP kinase, are not mitogenic and do not induce p44^{mapk} nuclear translocation. Thus it is likely that this discrepancy between our results and theirs is due to a marked difference in the response of CCL39 and HeLa cells to phorbol esters. This observation strengthens our previous findings that MAP kinase activation appears to be essential to deliver the mitogenic response (Pagès et al., 1993) and we propose that nuclear transfer is critical for MAP kinase action. However this notion is not general since in dog thyrocytes the cAMP pathway that leads to mitogenicity does so without activating MAP kinases (Lamy et al., 1993). Nevertheless, we predict that the EGF-induced mitogenicity should require MAP kinase activation in these cells.

Transfected p44^{mapk} and p42^{mapk} showed marked accumulation in the nucleus of nonstimulated cells and in particular when overexpressed. Thus, one possibility is that overexpression overwhelms the capacity of a putative cytoplasmic anchor to retain MAP kinases in the cytoplasm. Finally, the expression of MAP kinase-dead mutants yielded an unexpected result. We found that the nuclear transfer as well as the retention in the nucleus were independent of the state of MAP kinase activity. This observation implicates that the nuclear transport is achieved irrespective of kinase phosphorylation/activation. This is confirmed by the nuclear localization of overexpressed wild type MAP kinases that are neither active nor phosphorylated in the G0 state. The mechanism of MAP kinase nuclear transfer is obscure since MAP kinases do not seem to possess any canonical nuclearization signal in their sequence. However it was possible to abolish the growth factor-stimulatable as well as constitutive transfer into the nucleus by removing the last 129 residues of p44^{mapk}. This approach will be refined to map the domains critical for nuclear transfer.

A second aspect of the present study demonstrated for the first time that the MAP kinase activator, p45^{mapkk}, remained cytoplasmic during the cell cycle regardless of its state of activation and even when overexpressed. Thus, unlike MAP kinases, p45^{mapkk} appears to be totally excluded from the nucleus. This finding is rather intriguing if we accept that the upstream activator of p45^{mapkk}, raf-1 also translocates to the nucleus in response to growth factors (Rapp et al., 1988). Why is the entire protein kinase module not nuclear? An alternative interpretation is that raf-1, like MAP kinases exerts a specific function in the nucleus modulating transcription factors. In that context a cytoplasmic MAP kinase kinase activator, distinct of raf-1, should exist. This is precisely the case since a 78-kD protein kinase capable to phosphorylate and activate p45^{mapkk} has just been characterized (Lange-Carter et al., 1993). This protein kinase referred to as MAP kinase kinase kinase is the homolog of byr2 of *S. pombe*. We predict that like p45^{mapkk}, this activator should have a cytoplasmic localization. If this model is correct, how can the MAP kinases present in the nucleus remain active if their activator, p45^{mapkk}, is excluded from the nucleus? One has to

postulate that MAP kinases shuttle between the nucleus and the cytoplasm to stay continuously activated, or that phosphatase activity is differentially regulated in the cytoplasm and the nucleus. Alternatively, a partial cotransfer to the nucleus of p45^{mapkk} complexed with its substrate, MAP kinases could have easily escaped our technique of immunostaining.

In summary, we previously demonstrated that MAP kinase activation is required for growth factor-stimulated cells to progress and enter S-phase. Here we have shown that MAP kinases specifically translocate to the nucleus and remain nuclear during the entire G1 period, a process, we believe, essential to induce gene expression. Future experiments will be designed to inhibit specifically MAP kinase nuclear translocation, a mean that should lead to cell growth arrest.

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References

- Ahn, N. G., R. Seger, R. L. Bratlien, C. D. Diltz, N. K. Tonks, and E. G. Krebs. 1991. Multiple components in an epidermal growth factor-stimulated protein kinase cascade. *J. Biol. Chem.* 266:4220-4227.
- Alvarez, E., I. C. Northwood, F. A. Gonzalez, D. A. Latour, A. Seth, C. Abate, T. Curran, and R. J. Davis. 1991. Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate protein phosphorylation. Characterization of the phosphorylation of c-Myc and c-Jun proteins by an epidermal growth factor threonine 669 protein kinase. *J. Biol. Chem.* 266:15277-15285.
- Anderson, N. G., J. L. Maller, N. K. Tonks, and T. W. Sturgill. 1990. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature (Lond.)* 343:651-653.
- Baker, S. J., T. K. Kerppola, D. Luk, M. T. Vandenberg, D. R. Marshak, T. Curran, and C. Abate. 1992. Jun is phosphorylated by several protein kinases at the same sites that are modified in serum-stimulated fibroblasts. *Mol. Cell Biol.* 12:4694-4705.
- Boulton, T. G., S. H. Nye, D. J. Robbins, N. Y. Ip, E. Radziejewska, S. D. Morgenbesser, R. A. DePinho, N. Panayotatos, M. H. Cobb, and G. D. Yancopoulos. 1991. ERKs; a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell.* 65:663-675.
- Chen, R.-H., C. Sarnecki, and J. Blenis. 1992. Nuclear localization and regulation of erk and rsk-encoded protein kinases. *Mol. Cell Biol.* 12:915-927.
- Courchesne, W. E., R. Kunisawa, and J. Thorner. 1989. A putative protein kinase overcomes pheromone-induced arrest of cell cycling in *S. cerevisiae*. *Cell.* 58:1107-1119.
- Dent, P., W. Haser, T. A. J. Haystead, L. A. Vincent, T. M. Roberts and T. W. Sturgill. 1992. Activation of mitogen activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. *Science (Wash. DC)* 257:1404-1407.
- Elion, E. A., P. L. Grisafi, and G. R. Fink. 1990. Fus3 encodes a cdc2/cdc28-related kinase required for the transition from mitosis into conjugation. *Cell.* 60:649-664.
- Gille, H., A. D. Sharrocks, and P. E. Shaw. 1992. Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-Fos promoter. *Nature (Lond.)* 358:414-421.
- Her, J., J. Wu, T. B. Rall, T. W. Sturgill, and M. J. Weber. 1991. Sequence of pp42/MAP kinase, a serine/threonine kinase regulated by tyrosine phosphorylation. *Nucleic Acids Res.* 19:3743.
- Kahan, C., K. Seuwen, S. Meloche, and J. Pouyssegur. 1992. Coordinate, biphasic activation of p44 mitogen activated protein kinase and S6 kinase by growth factors in hamster fibroblasts. *J. Biol. Chem.* 267:13369-13375.
- Kosako, H., Y. Gotoh, S. Matsuda, M. Ishikawa, and E. Nishida. 1992. Xenopus activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2903-2908.

- Kosako, H., E. Nishida, and Y. Gotoh. 1993. cDNA cloning of MAP kinase reveals kinase cascade pathways in yeasts to vertebrates. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:787-794.
- Kreis, T. E. 1986. Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:931-941.
- Kyriakis, J. M., H. App, X. Zhang, P. Banerjee, D. L. Brautigan, U. R. Rapp, and J. Avruch. 1992. Raf-1 activates MAP kinase-kinase. *Nature (Lond.)*. 358:417-421.
- L'Allemain, G., J. Her, R. L. Del Vecchio, and M. J. Weber. 1992a. Functional expression in mammalian cells of a full length cDNA coding for the pp42/MAP kinase (p42^{mapk}) protein. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 292:191-195.
- L'Allemain, G., J. Her, J. Wu, T. W. Sturgill, and M. J. Weber. 1992b. Growth factor-induced activation of a kinase activity which causes regulatory phosphorylations of p42/microtubule-associated protein kinase. *Mol. Cell. Biol.* 12:2222-2229.
- Lamy, F., F. Wilkin, M. Baptist, J. Posada, P. Roger, and J. E. Dumont. 1993. Phosphorylation of mitogen-activated protein kinases is involved in the epidermal growth factor and phorbol ester, but not in the thyrotropin/cAMP, thyroid mitogenic pathway. *J. Biol. Chem.* 268:8398-8401.
- Lange-Carter, C. A., C. M. Pleiman, A. M. Gardner, K. J. Blumer, and G. L. Johnson. 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science (Wash. DC)*. 260:315-319.
- Méloche, S., K. Seuwen, G. Pagès, and J. Pouyssegur. 1992a. Biphasic and synergistic activation of p44^{mapk} (ERK1) by growth factors: correlation between late phase activation and mitogenicity. *Mol. Endocrinol.* 6:845-854.
- Méloche, S., G. Pagès, and J. Pouyssegur. 1992b. Functional expression and growth factor activation of an epitope-tagged p44 mitogen-activated protein kinase, p44^{mapk}. *Mol. Biol. Cell.* 3:63-71.
- Nakiely, S., P. Cohen, J. Wu, and T. Sturgill. 1992. Map kinase activator from insulin-stimulated skeletal muscle is a protein threonine/tyrosine kinase. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2123-2129.
- Neiman, A. M., B. J. Stevenson, H.-P. Xu, G. F. Sprague, I. Herskowitz, M. Wigler, and S. Marcus. 1993. Functional homology of protein kinases required for sexual differentiation in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* suggest a conserved signal transduction module in eukaryotic organisms. *Mol. Biol. Cell.* 4:107-120.
- Nielsen, O. 1993. Signal transduction during mating and meiosis in *S. pombe*. *Trends Cell. Biol.* 3:60-65.
- Northwood, I. C., F. A. Gonzalez, M. Wartmann, D. L. Raden, and R. J. Davis. 1991. Isolation and characterization of two growth factor-stimulated protein kinases that phosphorylate the epidermal growth factor receptor at threonine 669. *J. Biol. Chem.* 266:15266-15276.
- Pagès, G., P. Lenormand, G. L'Allemain, J.-C. Chambard, S. Méloche, and J. Pouyssegur. 1993. The mitogen activated protein kinases p42^{mapk} and p44^{mapk} are required for fibroblast cell proliferation. *Proc. Natl. Acad. Sci. USA*. In press.
- Pelech, S. L., and J. S. Sanghera. 1992. Mitogen-activated protein kinases: versatile transducers for cell signaling. *Trends Biochem. Sci.* 17:233-238.
- Posada, J., and J. A. Cooper. 1992. Requirements for phosphorylation of MAP kinase during meiosis in *Xenopus* oocytes. *Science (Wash. DC)*. 255:212-215.
- Pouyssegur, J., and K. Seuwen. 1992. Transmembrane receptors and intracellular pathways that control cell proliferation. *Annu. Rev. Physiol.* 54:195-210.
- Pulverer, B. J., J. M. Kyriakis, J. Avruch, E. Nikolakaki, and J. R. Woodgett. 1991. Phosphorylation of c-Jun mediated by MAP kinases. *Nature (Lond.)*. 353:670-674.
- Rapp, U. R., G. Heidecker, M. Huleihel, J. L. Cleveland, W. C. Choi, T. Pawson, J. N. Ihle, and W. B. Anderson. 1988. Raf family serine/threonine protein kinases in mitogen signal transduction. *Cold Spring Harbor Symp. Quant. Biol.* LIII:173-184.
- Ray, L. B., and T. W. Sturgill. 1988. Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine in vivo. *Proc. Natl. Acad. Sci. USA*. 85:3753-3757.
- Roberts, T. M. 1992. A single chain of events. *Nature (Lond.)*. 360:534-535.
- Sanghera, J. S., M. Peter, E. A. Nigg, and S. L. Pelech. 1992. Immunological characterization of avian MAP kinases: evidence for nuclear localization. *Mol. Biol. Cell.* 3:775-787.
- Seger, R., D. Seger, F. J. Lozeman, N. G. Ahn, L. M. Graves, J. S. Campbell, L. Ericsson, M. Harrylock, A. M. Jensen, and E. G. Krebs. 1992. Human T-cell mitogen-activated protein kinase kinases are related to yeast signal transduction kinases. *J. Biol. Chem.* 267:25628-25631.
- Seth, A., F. A. Gonzalez, S. Gupta, D. L. Raden, and R. J. Davis. 1992. Signal transduction within the nucleus by mitogen activated protein kinase. *J. Biol. Chem.* 267:24796-24804.
- Sturgill, T. W., and J. Wu. 1991. Recent progress in characterization of protein kinase cascades for phosphorylation of ribosomal protein S6. *Biochim. Biophys. Acta.* 1092:350-357.
- Sturgill, T. W., L. B. Ray, E. Erikson, and J. L. Maller. 1988. Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature (Lond.)*. 334:715-718.
- Toda, T., M. Shimanuki, and M. Yanagida. 1991. Fission genes that confer resistance to staurosporin encode an AP-1 like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases. *Genes Dev.* 5:60-73.
- Torres, L., H. Martin, M. I. Garcia-Saez, J. Arroyo, M. Molina, M. Sanchez, and C. Nombela. 1991. A protein kinase gene complements the lytic phenotype of *Saccharomyces cerevisiae* *lyt2* mutants. *Mol. Microbiol.* 5:2845-2854.
- Vouret-Craviari, V., E. Van Obberghen-Shilling, U. B. Rassmussen, A. Pavirani, J.-P. Lecocq, and J. Pouyssegur. 1992. Synthetic α -thrombin receptor peptides activate G-protein coupled signalling pathways but are unable to induce mitogenesis. *Mol. Biol. Cell.* 3:95-102.
- Vouret-Craviari, V., E. Van Obberghen-Shilling, J. C. Scimeca, E. Van Obberghen, and J. Pouyssegur. 1993. Differential activation of p44^{mapk} (ERK1) by α -thrombin and thrombin-receptor peptide agonist. *Biochem. J.* 289:209-214.