

# Serum-induced Translocation of Mitogen-activated Protein Kinase to the Cell Surface Ruffling Membrane and the Nucleus

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**Abstract.** The mitogen-activated protein (MAP) kinase signal transduction pathway represents an important mechanism by which growth factors regulate cell function. Targets of the MAP kinase pathway are located within several cellular compartments. Signal transduction therefore requires the localization of MAP kinase in each sub-cellular compartment that contains physiologically relevant substrates. Here, we show that serum treatment causes the translocation of two human MAP kinase isoforms, p40<sup>mapk</sup> and p41<sup>mapk</sup>, from the cytosol into the nucleus. In addition, we report that p41<sup>mapk</sup> (but not p40<sup>mapk</sup>) is localized at the cell surface ruffling membrane in serum-treated cells.

To investigate whether the protein kinase activity of MAP kinase is required for serum-induced redistribu-

tion within the cell, we constructed mutated kinase-negative forms of p40<sup>mapk</sup> and p41<sup>mapk</sup>. The kinase-negative MAP kinases were not observed to localize to the cell surface ruffling membrane. In contrast, the kinase-negative MAP kinases were observed to be translocated to the nucleus. Intrinsic MAP kinase activity is therefore required only for localization at the cell surface and is not required for transport into the nucleus.

Together, these data demonstrate that the pattern of serum-induced redistribution of p40<sup>mapk</sup> is different from p41<sup>mapk</sup>. Thus, in addition to common targets of signal transduction, it is possible that these MAP kinase isoforms may differentially regulate targets located in distinct sub-cellular compartments.

CELLULAR proliferation and differentiation is regulated by the action of growth factors that bind to specific receptors expressed at the surface of responsive cells. Growth factor binding to these receptors initiates multiple signal transduction pathways that control cell physiology. One mechanism of signal transduction by growth factor receptors is represented by the mitogen-activated protein (MAP)<sup>1</sup> kinase pathway (2, 15, 19, 46). In the case of signal transduction by the EGF receptor it has been demonstrated that this pathway is complex and consists of multiple steps including the adaptor protein (GRB2/SEM-5 (38, 45), a guanine nucleotide exchange protein (41), *Ras* (36, 55, 58), and a protein kinase cascade involving a MAP kinase kinase kinase (*c-Raf-1* [21, 32, 34], *c-Mos* [47], *Stell* [35], or other protein kinase [26, 40]), MAP kinase kinase (5, 18, 33, 50, 56, 59), and MAP kinase (2, 15, 19, 46).

Growth factor-activated MAP kinase phosphorylates substrate proteins in vivo. These substrates represent targets of

the MAP kinase signal transduction pathway (19). In vitro analysis has demonstrated that MAP kinase exhibits a restricted substrate specificity (3, 14, 19, 27). However, several MAP kinase substrates have been identified (19). These substrates include: (a) cell surface proteins such as the EGF receptor (44, 53) and phospholipase A<sub>2</sub> (37, 43); (b) the cytoskeletal proteins tau (22) and caldesmon (12); and (c) the nuclear proteins *c-Jun* (3, 13, 48), *c-Myc* (3, 51, 52), NF-IL6 (42), ATF-2 (1), *TALI* (11), and p62<sup>TCF</sup>/*Elk-1* (25, 39). The targets of the MAP kinase signal transduction pathway are therefore located in the nucleus, the cytoplasm and at the cell surface. Analysis of sub-cellular distribution has demonstrated the presence of MAP kinase within the cytoplasm and the nucleus, but not at the cell surface (3, 10, 49, 52). A fundamental question about the process of signal transduction is therefore the identity of the mechanism that allows MAP kinase to couple to targets localized at the cell surface and within different cellular compartments.

The purpose of this study was to investigate the sub-cellular localization of the MAP kinase isoforms p40<sup>mapk</sup> and p41<sup>mapk</sup>. We report that these MAP kinases are located in the cytosol of serum-starved cells. However, serum treatment causes translocation of these MAP kinases from the

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1. Abbreviation used in this paper: MAP, mitogen-activated protein.

cytosol into the nucleus. Significantly, p41<sup>mapk</sup> (but not p40<sup>mapk</sup>) was also observed to be translocated to the cell surface ruffling membrane. The difference between the serum-induced redistribution of p40<sup>mapk</sup> and p41<sup>mapk</sup> suggests that these MAP kinase isoforms may be coupled to distinct targets of signal transduction in addition to common signaling pathways.

## Materials and Methods

### Plasmids

The plasmids pCH110 and pUC13 were from Pharmacia-LKB Biotechnology Inc. (Piscataway, NJ). The plasmid pGEM-Luc was from Promega (Madison, WI). The plasmids pGAL4/Myc and pG5E1bLuc have been described previously (3, 52). The plasmid pMyc contains the human *c-myc* gene under the control of an SV-40 promoter (30). The plasmid pG5E1bLuc has been described previously (52).

The plasmid pCMV-p41<sup>mapk</sup> was constructed using a 1,992-bp *EspI* restriction fragment of the human p41<sup>mapk</sup> cDNA (28) (available from GenBank under accession number Z11694) cloned as a blunt-ended fragment in the *SmaI* site of the polylinker of the expression vector pCMV5 (4). The protein sequence of human p41<sup>mapk</sup> has a calculated molecular mass of 41 kD and is 98.3% identical to the rat ERK2 protein kinase (7, 28). Mutation of p41<sup>mapk</sup> at the ATP binding site to inhibit protein kinase activity was performed by replacing Lys<sup>54</sup> and Lys<sup>55</sup> (sequence AAGAAA) in sub-region II (according to the nomenclature of Hanks et al. [31]) with two Ala residues (sequence GCGGCA) using a polymerase chain reaction-based procedure (54) to create the plasmid pCMV-p41(Ala<sup>54</sup>Ala<sup>55</sup>)<sup>mapk</sup>.

The plasmid pCMV-p40<sup>mapk</sup> was constructed using a 1,940-bp *SacII-EspI* restriction fragment of the human p40<sup>mapk</sup> cDNA (28) (available from GenBank under accession number Z11695) cloned as a blunt-ended fragment in the *SmaI* site of the polylinker of the expression vector pCMV5. The protein sequence of human p40<sup>mapk</sup> has a calculated molecular mass of 40 kD and is 96.8% identical to the rat ERK2 protein kinase (7, 28). Mutation of p40<sup>mapk</sup> at the ATP binding site to inhibit protein kinase activity was performed by replacing Lys<sup>42</sup> and Lys<sup>43</sup> (sequence AAGAAA) in sub-region II with Ala residues (sequence GCGGCA) using a polymerase chain reaction-based procedure (54) to create the plasmid pCMV-p40(Ala<sup>42</sup>Ala<sup>43</sup>)<sup>mapk</sup>.

### Cell Culture and Transfection Assays

COS-7 cells were subcultured in DME supplemented with 5% FBS (GIBCO BRL, Gaithersburg, MD). Transfections were performed using the DEAE-Dextran method as previously described (3). The cells were trypsinized 20 h after transfection, seeded into 100-mm dishes or 4-well tissue culture chambers mounted on a glass slide (Lab-tek, Naperville, IL), and incubated in DME containing 5% FBS for 28 h. Serum-starved cells were transferred to medium without serum during the last 18 h of incubation. Serum stimulation was performed by adding DME containing 10% FBS.

### Measurement of Myc-directed Transactivation of Reporter Gene Expression

Transactivation assays were performed using 0.02  $\mu$ g of pGAL4/Myc activator plasmid and 2  $\mu$ g of a luciferase reporter plasmid (pG5E1bLuc) as previously described (51). Normalization of transfection efficiency was performed using a control plasmid (1  $\mu$ g of pCH110) that expresses  $\beta$ -galactosidase. To investigate the effect of MAP kinase expression, the cells were transfected with 2  $\mu$ g of pCMV5 (vector control), pCMV-p40<sup>mapk</sup>, pCMV-p40(Ala<sup>42</sup>Ala<sup>43</sup>)<sup>mapk</sup>, pCMV-p41<sup>mapk</sup>, or pCMV-p41(Ala<sup>54</sup>Ala<sup>55</sup>)<sup>mapk</sup>. The total DNA in all transfections was maintained at 10  $\mu$ g using pUC13 as carrier DNA. The cells were harvested 48 h after transfection and cell extracts were prepared as previously described (51). Luciferase activity was measured using 2.5  $\mu$ l of the cell extract with a luminometer (model 2010; Analytical Luminescence Laboratory, San Diego, CA) (51).  $\beta$ -Galactosidase activity was measured by mixing 15  $\mu$ l of the cell extract with 3  $\mu$ l of 0.1 M MgCl<sub>2</sub>/4.5M  $\beta$ -mercaptoethanol, 66  $\mu$ l of 4 mg/ml *o*-nitrophenyl- $\beta$ -D-galactopyranoside (Sigma Immunochemicals, St. Louis, MO) and 216  $\mu$ l of 0.1 M sodium phosphate, pH 7.5. The incubations were performed at 37°C for 5 min or until a faint yellow color appeared. The reactions were stopped by adding 500  $\mu$ l of 1 M Na<sub>2</sub>CO<sub>3</sub> to each tube and

the optical density at 420 nm was measured using a spectrophotometer (Pharmacia-LKB Biotechnology Inc.).

### Isolation of Protein Kinases

The protein kinases p40<sup>mapk</sup> and p41<sup>mapk</sup> were isolated from transfected COS-7 cells by sequential chromatography on Mono-Q and phenyl-Superose columns (Pharmacia-LKB Biotechnology, Inc.) as described previously (44).

### MAP Kinase Assays

Protein kinase activity was measured using a synthetic peptide substrate based on the sequence of the EGF receptor surrounding the Thr<sup>669</sup> phosphorylation site (16, 44, 53) as described by Gonzalez et al. (27). Transfected COS-7 cells were lysed at 4°C in 25 mM Hepes, pH 7.4, 5 mM EDTA, 50 mM NaF, 100  $\mu$ M sodium orthovanadate, 1 mM PMSF and 10  $\mu$ g/ml leupeptin. The lysate was cleared by centrifugation at 100,000 *g* for 20 min at 4°C and the supernatant containing that MAP kinase activity was collected. Phosphorylation assays were performed using 25 mM Hepes, pH 7.4, 10 mM MgCl<sub>2</sub>, 50  $\mu$ M ATP[ $\gamma$ -<sup>32</sup>P] (10 Ci/mmol), 1 mg/ml synthetic peptide, and 10  $\mu$ l of kinase preparation in a final volume of 25  $\mu$ l. Reactions were terminated after 20 min at 22°C by the addition of 10  $\mu$ l of 95% formic acid containing 75 mM ATP. The phosphorylated synthetic peptide was isolated by applying 25  $\mu$ l of the reaction mixture onto phosphocellulose paper (P81; Whatman Inc., Clifton, NJ) and washing the filters twice in 1 M acetic acid, 4 mM sodium pyrophosphate for 20 min. Radioactivity was quantitated by measuring Cerenkov radiation with a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Nonspecific incorporation of radioactivity was determined in incubations without synthetic peptide substrate.

### Western Blot Analysis

Cells were solubilized in lysis buffer (25 mM Hepes, pH 7.5, 5 mM EDTA, 1 mM PMSF and 10  $\mu$ g/ml leupeptin) and the extracts were centrifuged at 100,000 *g* for 20 min at 4°C. The supernatant was subjected to SDS-PAGE and the resolved proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA). The membranes were then probed with a mouse anti-MAP/ERK kinase monoclonal antibody (Zymed Laboratories, Inc., San Francisco, CA) to detect the MAP kinases. The immune complexes were visualized with Kodak X-O-MAT AR film (Eastman Kodak Co., Rochester, NY) using enhanced chemiluminescence detection (Amersham International, Buckinghamshire, England) or <sup>125</sup>I-goat anti-mouse Ig (Dupont-NEN). Quantitative detection of the radioactivity was performed using a Phosphorimager and ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA).

### Stoichiometry of EGF Receptor Phosphorylation

COS cells seeded in 35-mm dishes were labeled with [<sup>32</sup>P]phosphate by incubation with phosphate-free modified Eagle's medium (Flow Laboratories, Inc., McLean, VA) supplemented with 0.1% calf serum (GIBCO-BRL) and 2 mCi/ml [<sup>32</sup>P]phosphate (Dupont-NEN, Boston, MA). The cells were treated with 10% FBS for defined times. The cells were then lysed with 25 mM Hepes, pH 7.4, 1% (vol/vol) Triton X-100, 1% (wt/vol) Na deoxycholate, 0.1% Na dodecylsulphate, 50 mM Na fluoride, 5 mM EDTA, 10  $\mu$ g/ml leupeptin, and 1 mM PMSF. The EGF receptors were isolated by immunoprecipitation using a rabbit polyclonal anti-EGF receptor antibody and subjected to polyacrylamide gel electrophoresis. The EGF receptor was then eluted from the gel and digested with trypsin (8, 20). The tryptic [<sup>32</sup>P]phosphopeptides obtained were resolved by reverse-phase high pressure liquid chromatography to isolate the peptide phosphorylated at Thr-669 (8, 20). The stoichiometry of phosphorylation of the EGF receptor was determined as described previously (8, 20). Calculation of the stoichiometry of the phosphorylation of the EGF receptor requires knowledge of both the number of EGF receptors and the specific radioactivity of the phosphate incorporated into the receptor. EGF receptor number was estimated by Western blotting using a polyclonal anti-receptor antibody using <sup>125</sup>I-protein A (Dupont-NEN) and detection with a Phosphorimager (Molecular Dynamics Inc.). The Western blotting procedure was quantitated by comparison with different amounts of plasma membranes electrophoresed on the same gel and transferred to nitrocellulose. EGF receptor number in the plasma membranes was measured using a radioreceptor assay using <sup>125</sup>I-EGF (8, 20). The specific radioactivity of the  $\gamma$ -phosphate of ATP was measured by the technique of England and Walsh (23).

## Immunocytochemistry

Transfected cells were plated into 4-well tissue culture chambers mounted on a glass slide (Lab-tek) and incubated for 10 h in DME supplemented with 5% FBS. To examine the effect of serum on the cellular distribution of MAP kinases, the cells were washed and incubated in serum-free DME for 18 h, and then treated without or with 10% FBS (GIBCO-BRL) for 30 min at 37°C. The cells were washed and processed for immunofluorescence in KRH buffer (120 mM NaCl, 6 mM KCl, 1.2 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 25 mM Hepes, pH 7.4).

The cells were fixed in KRH containing 3.7% formaldehyde (15 min, 22°C), washed and incubated in KRH containing 50 mM NH<sub>4</sub>Cl for 15 min at 22°C. Then, they were permeabilized with 0.2% Triton X-100 in KRH (5 min, 22°C), washed three times and incubated for 1 h in KRH containing the primary antibody and 20% horse serum at 22°C. The primary antibodies and the dilutions used were: (a) a mouse monoclonal anti-c-Myc antibody (Ab-2; Oncogene Science, Inc., Manhasset, NY) diluted 1:20; (b) a mouse monoclonal anti-MAP/ERK kinase antibody (Zymed Laboratories, Inc.) diluted 1:500; and (c) a sheep polyclonal anti-EGF receptor antibody diluted 1:200. The anti-EGF receptor antibody was purified from serum that was obtained after immunization of a sheep with a bacterially expressed carboxyl-terminal fragment of the human EGF receptor as an antigen.

After the incubation with primary antibodies, the cells were washed three times and incubated for 1 h at 22°C in KRH containing secondary antibodies and 20% horse serum. The secondary antibodies and the dilutions used were: (a) a rhodamine-conjugated rabbit anti-mouse Ig antibody (Boehringer Mannheim Corp., Indianapolis, IN) diluted 1:500; (b) an FITC-conjugated goat anti-mouse Ig antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) diluted 1:300; and (c) an FITC-conjugated rabbit anti-sheep Ig antibody (ICN Immunochemicals, Costa Mesa, CA) diluted 1:500. Actin filaments were stained with 5 U/ml of rhodamine-labeled phalloidin (Molecular Probes, Inc., Eugene, OR) during the incubation with the secondary antibody. The cells were then washed with KRH and mounted onto slides with a solution of 87.5% glycerol, 10% PBS, and 2.5% DABCO. Beads (200 nm) with a broad fluorescent emission (Molecular Probes, Inc.) were added to the mounting medium as fiducial markers.

Control experiments were performed to assess the specificity of each antibody used for indirect immunofluorescence experiments. No fluorescence was detected using the secondary antibodies in the absence of primary antibody. To confirm the presence of ruffling membranes after serum stimulation we stained the actin filaments in the cells with rhodamine-labeled phalloidin. FITC-conjugated WGA (Molecular Probes, Inc.) was also used to visualize the cell surface ruffling membrane. It was found that the expression of both the wild-type and kinase-negative forms of the MAP kinases p40<sup>mapk</sup> and p41<sup>mapk</sup> had no significant effect on the ability of serum to induce the formation of the cell surface membrane ruffles.

## Digital Imaging Microscopy and Image Restoration

Cells are complex three-dimensional objects. The image from a single focal plane acquired using a conventional fluorescence microscope is degraded by light originating above and below the plane of interest. Therefore, details that are contained within a two-dimensional image of a cell may be obscured by the out of focus light. To minimize this problem, we used image restoration procedures to achieve increased contrast, higher resolution, and greater numerical accuracy (9, 24).

Digital images of the fluorescence distribution in single cells were obtained using a Nikon 60× Planapo objective (NA = 1.4) on a Zeiss IM-35 microscope equipped for epifluorescence as previously described (24). Images of various focal planes were obtained with a computer controlled focus mechanism and a thermoelectrically cooled charge coupled device camera (model 220; Photometrics Ltd., Tucson, AZ). The exposure of the sample to the excitation source was determined by a computer-controlled shutter and wavelength selector system (MVI, Avon, MA). The charge coupled device camera and microscope functions were controlled by a microcomputer, and the data acquired from the camera were transferred to a Silicon Graphics model 4D/GTX workstation (Mountainview, CA) for image processing. Images were corrected for non-uniformities in sensitivity and for the dark current of the charge coupled device detector. The calibration of the microscopy blurring was determined by measuring the instrument's point spread function as a series of optical sections at 0.125-μm intervals of a 0.19-μm diameter fluorescently labeled latex bead (Molecular Probes, Inc.). The image restoration algorithm used was based upon the theory of ill-posed problems and obtains quantitative dye density values within the cell that are substantially more accurate than those in an un-processed im-

age (9). After image processing, individual optical sections of cells were inspected and analyzed using computer graphics software on a Silicon Graphics workstation. Quantitative molecular density values within a single optical section of individual cells were estimated by defining 15 concentric regions where the first region enclosed the cell nucleus and the fifteenth region enclosed the outermost cell fluorescence but excluded extracellular background fluorescence. The fluorescence intensity values were normalized by calculating the ratio of the average-pixel intensity within each region to the average-pixel intensity over all 15 regions.

## Laser Scanning Confocal Microscopy

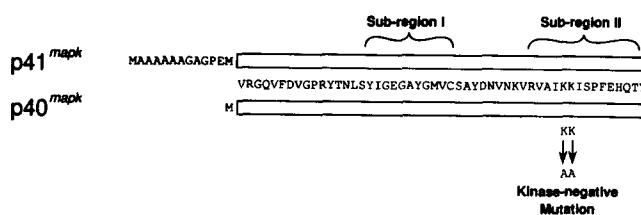
The confocal microscope, unlike standard fluorescent microscopes, provides an improved resolution along the z axis by rejecting out-of-focus fluorescence (57). Thus, to identify the subcellular location of the p41<sup>mapk</sup> protein we used fluorophore-labeled antibodies and a laser scanning confocal microscope (model MRC-600; Bio Rad Laboratories, Hercules, CA) equipped with a Nikon 60× oil immersion lens (NA = 1.4). Image sets consisting of at least 20 optical sections at 0.3-μm intervals were obtained for analysis. Each pseudocolor image represents an optical section that was acquired by accumulating four scans of the laser.

## Results

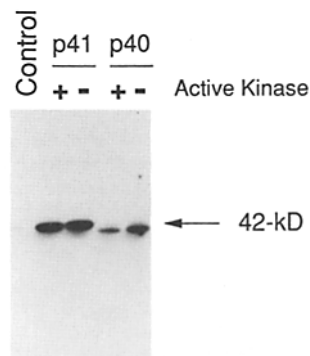
### Biochemical Characterization of p40<sup>mapk</sup> and p41<sup>mapk</sup>

Two isoforms of the human ERK2 protein kinase that are expressed by HeLa cells have been identified by molecular cloning (28). These protein kinases share a high degree of sequence identity with the exception that p41<sup>mapk</sup> has a short NH<sub>2</sub>-terminal extension compared with p40<sup>mapk</sup> (Fig. 1). To determine the significance of this difference in primary structure, we examined the properties of the two ERK2 isoforms expressed in cultured cells. Western blot analysis of the cell lysates detected a marked increase (~20-fold) in the level of expression of p40<sup>mapk</sup> and p41<sup>mapk</sup> (Fig. 2). Immunofluorescence analysis demonstrated that the transfection procedure resulted in the over-expression of MAP kinase in 15–20% of the COS cells. Thus, we can calculate that the over-expression of MAP kinase in the transfected cells was ~100-fold over the endogenous MAP kinase. This over-expression of p40<sup>mapk</sup> and p41<sup>mapk</sup> was associated with increased growth factor-stimulated MAP kinase activity detected in cell lysates (Fig. 3). Significantly, the mutation of the ERK2 isoforms within the conserved protein kinase sub-domain II (Fig. 1) resulted in the expression of kinase-negative forms of these MAP kinases (Figs. 2 and 3).

In an initial analysis of the biochemical properties of p40<sup>mapk</sup> and p41<sup>mapk</sup> we compared the chromatographic elution profiles of these protein kinases. A similar elution profile of p40<sup>mapk</sup> and p41<sup>mapk</sup> was observed from an anion



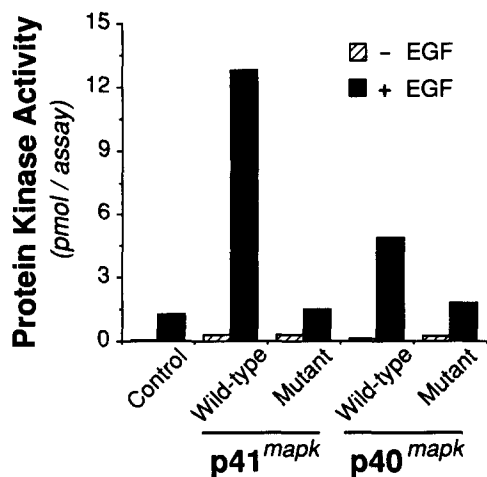
**Figure 1.** Comparison of the NH<sub>2</sub>-terminal region of p41<sup>mapk</sup> and p40<sup>mapk</sup>. The conserved protein kinase sub-regions I and II (31) are illustrated. Regions of identity are presented and boxed. Point mutations in sub-region II that result in the loss of protein kinase activity are presented below the protein sequence.



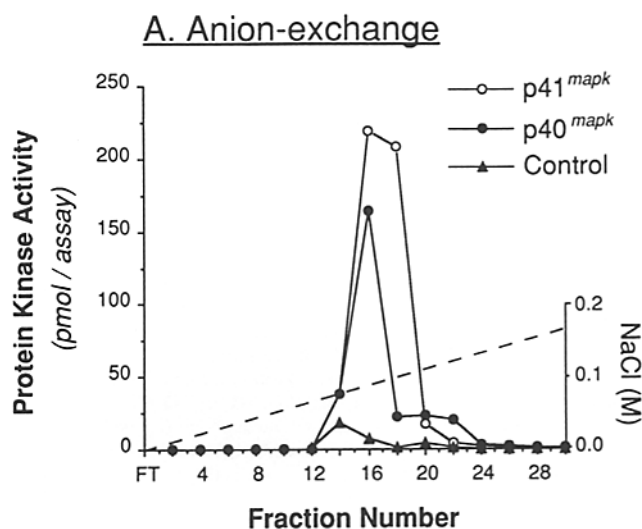
**Figure 2.** Expression of p41<sup>mapk</sup> and p40<sup>mapk</sup> in cultured cells. Extracts were prepared from COS-7 cells transfected with pCMV5 (Control), pCMV-p41<sup>mapk</sup>, pCMV-41 (Ala<sup>54</sup>Ala<sup>55</sup>)<sup>mapk</sup>, pCMV-p40<sup>mapk</sup>, or pCMV-p40(Ala<sup>42</sup>Ala<sup>43</sup>)<sup>mapk</sup>. MAP kinase expression was examined by Western blot analysis (50  $\mu$ g of the cell extract) using a monoclonal anti-MAP kinase antibody and the enhanced

chemiluminescence detection procedure. Quantitation of the Western blot was performed using <sup>125</sup>I-labeled protein A to detect the immune complexes and a Phosphorimager with ImageQuant software (Molecular Dynamics Inc.). The level of over-expression of the transfected MAP kinase was approximately 20-fold over the level of the endogenous MAP kinase. Similar data were obtained in three separate experiments.

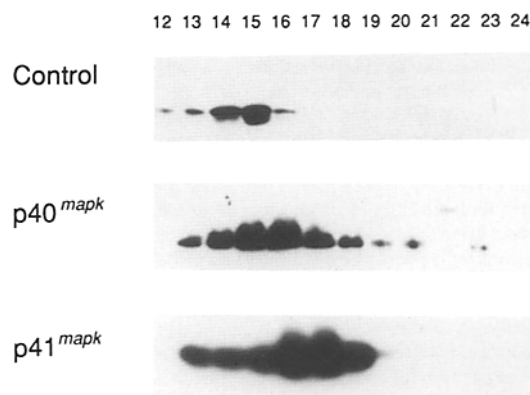
exchange column (Fig. 4). The partially purified protein kinases were then applied to a hydrophobic interaction column. This column was washed with high salt and the bound proteins were eluted using a decreasing salt gradient in the presence of ethylene glycol. A single peak of protein kinase activity was eluted from the column in each case. However, the elution of p40<sup>mapk</sup> from the hydrophobic interaction column was found to be modestly retarded in comparison to that of p41<sup>mapk</sup> (Fig. 5). Together, these data demonstrate that the chromatographic properties of p40<sup>mapk</sup> and p41<sup>mapk</sup> are very similar.



**Figure 3.** Expression of p41<sup>mapk</sup> and p40<sup>mapk</sup> causes an increase in MAP kinase activity. Extracts were prepared from COS-7 cells that were mock-transfected (Control) or transfected with plasmid vectors to express wild-type and kinase-negative forms of p40<sup>mapk</sup> and p41<sup>mapk</sup>. MAP kinase activity was measured using a synthetic peptide substrate based on the primary sequence of the EGF receptor surrounding the phosphorylation site Thr<sup>669</sup>. The results are presented as the mean of data obtained in three independent experiments.



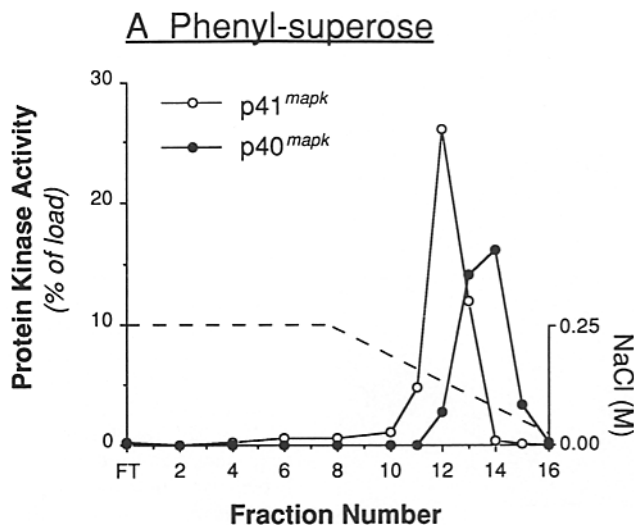
## B. Western Blot



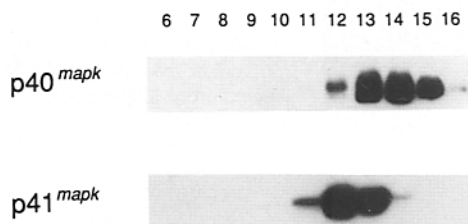
**Figure 4.** Anion exchange chromatography of p41<sup>mapk</sup> and p40<sup>mapk</sup>. 10 100 mM dishes of COS cells ( $\Delta$ ) expressing p40<sup>mapk</sup> ( $\bullet$ ) or p41<sup>mapk</sup> ( $\circ$ ) were treated with 100 nM EGF for 5 min. Soluble extracts were fractionated by analytical ion exchange chromatography using a Mono Q column as described under Materials and Methods. (A) Protein kinase activity in fractions eluted from the column was measured using the synthetic peptide KRELVE-PLT<sup>669</sup>PSGEAPNQALLR as substrate. The concentration of NaCl in the eluate is indicated (---). (B) Western blot analysis of MAP kinase elution from the Mono Q column. Fractions corresponding to the peak of protein kinase activity were analyzed by SDS-PAGE, transferred to Immobilon-P, and probed with MAP kinase antibody using enhanced chemiluminescence detection.

In further studies to compare the properties of p40<sup>mapk</sup> and p41<sup>mapk</sup>, we examined the effect of the expression of these kinases on a physiologically relevant target of the MAP kinase signal transduction pathway. In previous studies we have demonstrated that c-Myc is a substrate for phosphorylation by MAP kinase at Ser-62 and that the phosphorylation at this site positively regulates the c-Myc transactivation domain (51, 52). We therefore examined the effect of the expression of p40<sup>mapk</sup> and p41<sup>mapk</sup> on the c-Myc transactivation





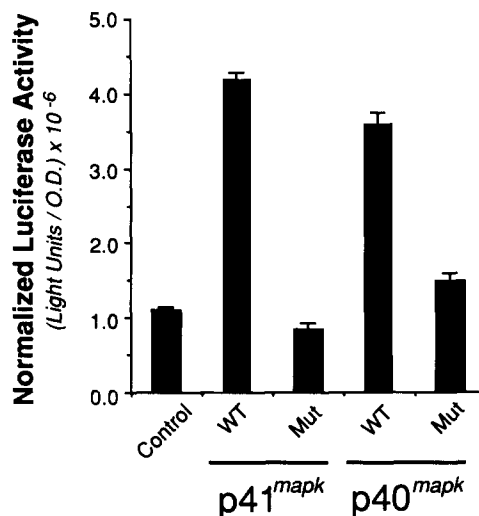
## B Western Blot



**Figure 5.** Phenyl-superose chromatography of  $p41^{mapk}$  and  $p40^{mapk}$ . Peak fractions of MAP kinase activity obtained after anion exchange chromatography (Fig. 4) were applied to a phenyl-Superose column. (A) Protein kinase activity of  $p40^{mapk}$  (●) and  $p41^{mapk}$  (○) was measured using the synthetic peptide KRELVEPLT<sup>669</sup>PSG-EAPNQALLR as a substrate. The protein kinase activity in the flow through (FT) and in the eluted fractions is presented. The concentration of NaCl in the eluate is indicated (---). (B) Fractions corresponding to the peak of protein kinase activity were analyzed by SDS-PAGE, transferred to Immobilon-P, and probed with MAP kinase antibody using enhanced chemiluminescence detection.

domain by measurement of the expression of a luciferase reporter gene. Fig. 6 shows that both  $p40^{mapk}$  and  $p41^{mapk}$  caused a marked increase in transactivation of the reporter gene. In contrast, the kinase-negative forms of these MAP kinases did not significantly alter the reporter luciferase activity (Fig. 4). Together, these data demonstrate that the MAP kinase isoforms  $p40^{mapk}$  and  $p41^{mapk}$  are biologically active when expressed in cultured cells.

The results of the biochemical characterization of  $p40^{mapk}$  and  $p41^{mapk}$  indicates that the properties of these protein kinases are similar (Figs. 2–6). This observation suggests that the function of these protein kinases in vivo may also be similar. However, this analysis does not take account of the important role of sub-cellular compartmentation in determining enzyme function in vivo. We therefore compared the



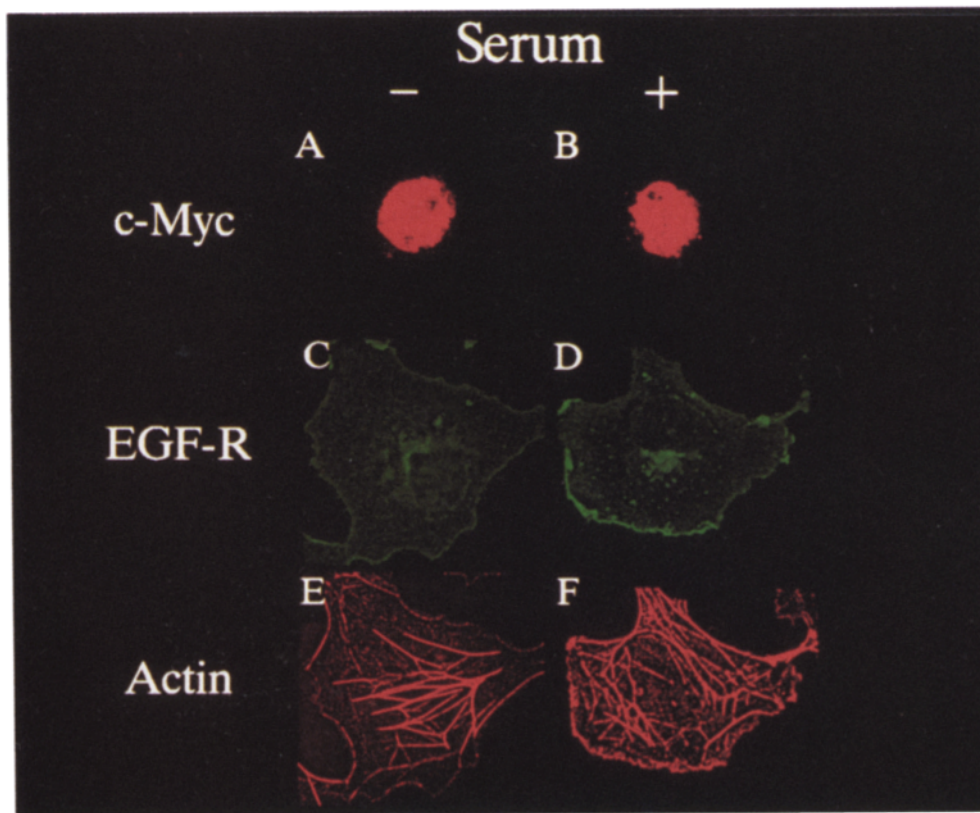
**Figure 6.** Effect of  $p41^{mapk}$  and  $p40^{mapk}$  on c-Myc-directed transactivation of reporter gene expression. The effect of transient expression of wild-type and mutated (kinase-negative) forms of  $p41^{mapk}$  and  $p40^{mapk}$  was investigated. COS-7 cells were co-transfected with 0.02  $\mu$ g of the activator plasmid pGAL4/Myc, 2  $\mu$ g of the reporter plasmid pG5E1BLuc and 1  $\mu$ g of a  $\beta$ -galactosidase expression vector (pCHI10) together with either 2  $\mu$ g of pCMV5 (Control), pCMV- $p41^{mapk}$ , pCMV- $p41^{Ala^{54}Ala^{55}mapk}$ , pCMV- $p40^{mapk}$ , or pCMV- $p40^{Ala^{42}Ala^{43}mapk}$ . The cells were grown in medium containing 5% FBS and extracts were prepared 48 h posttransfection. Differences in transfection efficiency were estimated by measurement of the  $\beta$ -galactosidase activity (OD value) in 5  $\mu$ l cell extract. Luciferase activities (light units) in 5  $\mu$ l of cell extract were measured and are presented as (Light Units/O.D.). Each data point represents the mean  $\pm$  S.E. of determinations obtained from three independent transfections. Similar data were obtained in three separate experiments.

cellular localization of  $p40^{mapk}$ ,  $p41^{mapk}$ , and substrates for these protein kinases.

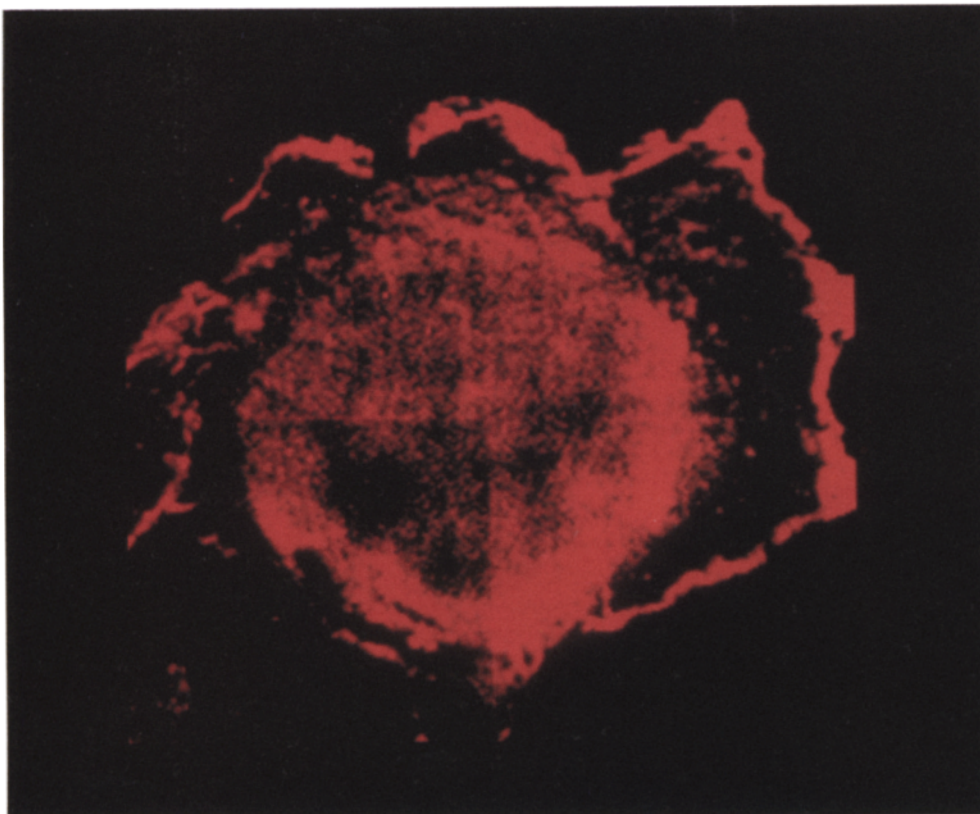
## Cellular Localization of MAP kinase Substrates

Several substrates for MAP kinase have been identified that are located in distinct subcellular compartments. The c-Myc protein is an example of a substrate that is found within the nucleus (3). Immunofluorescence, digital imaging microscopy, and image restoration procedures confirmed that the c-Myc protein was localized within the nucleus (Fig. 7). In contrast to the nuclear location of c-Myc, the EGF receptor is an example of a MAP kinase substrate that is present at the cell surface (16, 44, 53). Immunofluorescence analysis demonstrated that the EGF receptor was expressed at the cell surface with a relatively uniform distribution (Fig. 7).

In further experiments, the effect of serum treatment on the localization of these MAP kinase substrates was examined. It was found that serum caused no significant alteration in the nuclear location of c-Myc (Fig. 7). However, serum caused a redistribution of the EGF receptor to the cell surface ruffling membranes which were visualized by staining cellular actin with rhodamine-labeled phalloidin (Fig. 7). Together, these data demonstrate that MAP kinase substrates are located within several cellular compartments in serum-treated cells, including the nucleus and the cell surface



**Figure 7.** Localization of MAP kinase substrate proteins. Transfected COS-7 cells were serum-starved for 18 h and then treated without (A, C, and E) or with (B, D, and F) 10% FBS for 30 min. The cells were then fixed and analyzed by indirect immunofluorescence using a digital imaging microscope and image restoration techniques. Images were obtained for c-Myc (A and B) and the EGF receptor (C and D). Actin filaments were visualized using rhodamine-conjugated phalloidin (E and F). The figure presents representative images of the fluorescence detected from single cells.



**Figure 8.** Analysis of the distribution of endogenous MAP kinase. COS cells were treated with 10% FBS for 30 min. The cells were then fixed and analyzed by indirect immunofluorescence using a mouse monoclonal anti-MAP kinase antibody and a rhodamine-conjugated goat anti-mouse Ig antibody. The figure presents a representative image of the fluorescence detected from a single cell by digital imaging microscopy and processed for image restoration.

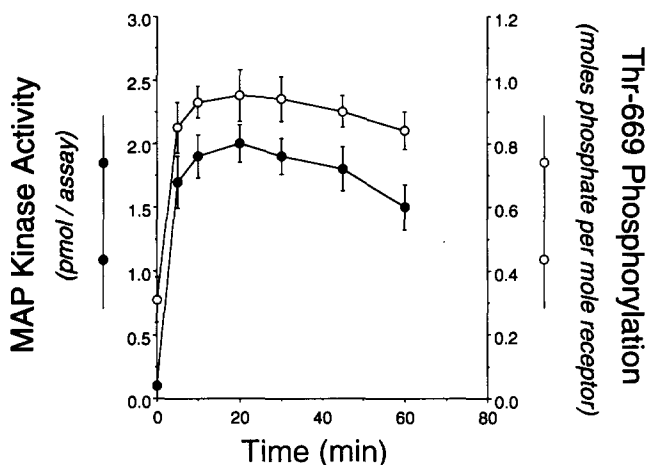
ruffling membranes. To account for the observed phosphorylation of these proteins *in vivo*, it is likely that MAP kinases are located within each of these cellular compartments.

We examined the distribution of the endogenous MAP kinases present in COS cells by immunofluorescence. Fig. 8 shows that the endogenous MAP kinases were detected at the cell surface, the perinuclear region, and within the nucleus of serum-treated cells. The cell surface and nuclear localization was observed within 5 min of serum treatment and was sustained for at least 1 h (data not shown). This time course is similar to the rapid and sustained activation of MAP kinase caused by serum in these cells (Fig. 9). Consistent with the presence of activated MAP kinase at the cell surface, a marked increase in EGF receptor phosphorylation at the MAP kinase site (Thr-669) was observed in serum-stimulated cells (Fig. 9).

Examination of MAP kinases present in COS cells demonstrates that these enzymes are present in several distinct sub-cellular compartments (Fig. 8). As this analysis does not distinguish between different MAP kinase isoforms, the distribution observed represents an average of all of the isoforms detected. It is possible that the sub-cellular localization of different MAP kinase isoforms may be distinct. Therefore, we examined the sub-cellular distribution of p40<sup>mapk</sup> and p41<sup>mapk</sup> using an over-expression strategy to distinguish between these individual MAP kinase isoforms and the endogenous MAP kinases present in COS cells (Figs. 10 and 11).

### Sub-cellular Localization of p40<sup>mapk</sup>

To investigate the localization of p40<sup>mapk</sup>, we performed immunofluorescence analysis using a monoclonal anti-MAP kinase antibody. The protein kinase p40<sup>mapk</sup> was visualized in the cytosol and the nuclear region of serum-starved cells (Fig. 10). Treatment of the cells with serum was associated with a decreased level of p40<sup>mapk</sup> in the cytosol and an increase in the level of p40<sup>mapk</sup> in the nucleus (Fig. 10). This



**Figure 9.** Comparison of the time course of MAP kinase activation and the phosphorylation of the EGF receptor at Thr-669. COS cells were treated with 10% serum for defined times. The MAP kinase activity (●) and the stoichiometry of phosphorylation of the EGF receptor at Thr-669 (○) were measured. The figure presents the results (mean ± SD) of three determinations.

observation implies that serum treatment induces the translocation of p40<sup>mapk</sup> from the cytosol into the nucleus.

To achieve a quantitative understanding of the sub-cellular distribution of p40<sup>mapk</sup> we analyzed the fluorescence images obtained by digital imaging microscopy. The method we used allowed the compilation of data from several individual cells and the calculation of the average fluorescence in discrete sub-cellular regions (Fig. 12). This quantitative analysis of the immunofluorescence demonstrated that the treatment of cells with serum caused a significant redistribution of cytosolic p40<sup>mapk</sup> to the nucleus (Figs. 12 and 13). Significantly, no apparent localization of p40<sup>mapk</sup> to the surface ruffling membrane of serum-treated cells was detected (Fig. 10).

### Sub-cellular Localization of p41<sup>mapk</sup>

The protein kinase p41<sup>mapk</sup> was detected in the cytosol of serum-starved cells (Fig. 11). A low level of p41<sup>mapk</sup> was also detected in the nuclear region (Fig. 11). Treatment of the cells with serum caused a marked increase in the nuclear localization of p41<sup>mapk</sup> and a corresponding decrease in the level of p41<sup>mapk</sup> in the cytosol (Fig. 11). In addition, it was observed that p41<sup>mapk</sup> was localized at the border of serum-treated cells. This location at the cell border coincided with the cell surface ruffling membranes which were visualized by staining with rhodamine-labeled phalloidin and FITC-conjugated WGA (data not shown). The cell surface and nuclear localization of p41<sup>mapk</sup> observed using the digital imaging microscope and image restoration procedures (Fig. 11) was also observed by analysis of the immunofluorescence by laser scanning confocal microscopy (Fig. 14). Quantitative analysis of the immunofluorescence demonstrated that serum caused a significant redistribution of p41<sup>mapk</sup> to the nuclear region and to the cell surface ruffling membrane (Figs. 12 and 13).

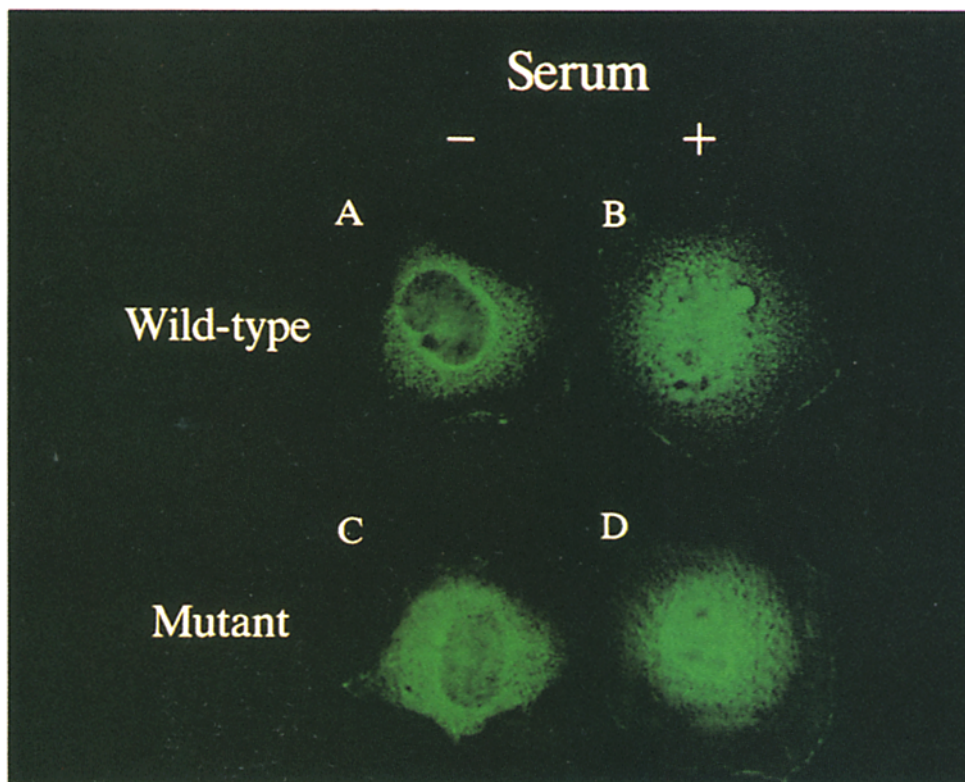
### MAP Kinase Activity Is Required for Redistribution to the Cell Surface, but not for Translocation to the Nucleus

The MAP kinase isoforms p40<sup>mapk</sup> and p41<sup>mapk</sup> were observed to redistribute from the cytosol to the nucleus after treatment of cells with serum (Figs. 10–13). The kinase-negative mutant forms of p40<sup>mapk</sup> and p41<sup>mapk</sup> also exhibited serum-stimulated translocation into the nucleus (Figs. 10–13). Thus, kinase activity is not required for the serum-induced nuclear translocation of p40<sup>mapk</sup> and p41<sup>mapk</sup>. In contrast, kinase activity was required for the serum-stimulated redistribution of MAP kinase to the cell surface ruffling membrane. Furthermore, the cell surface redistribution was found only for p41<sup>mapk</sup> and was not observed for p40<sup>mapk</sup> (Figs. 10 and 11). Together, these observations demonstrate that there are distinct structural requirements for the serum-stimulated redistribution of MAP kinases to different cellular compartments.

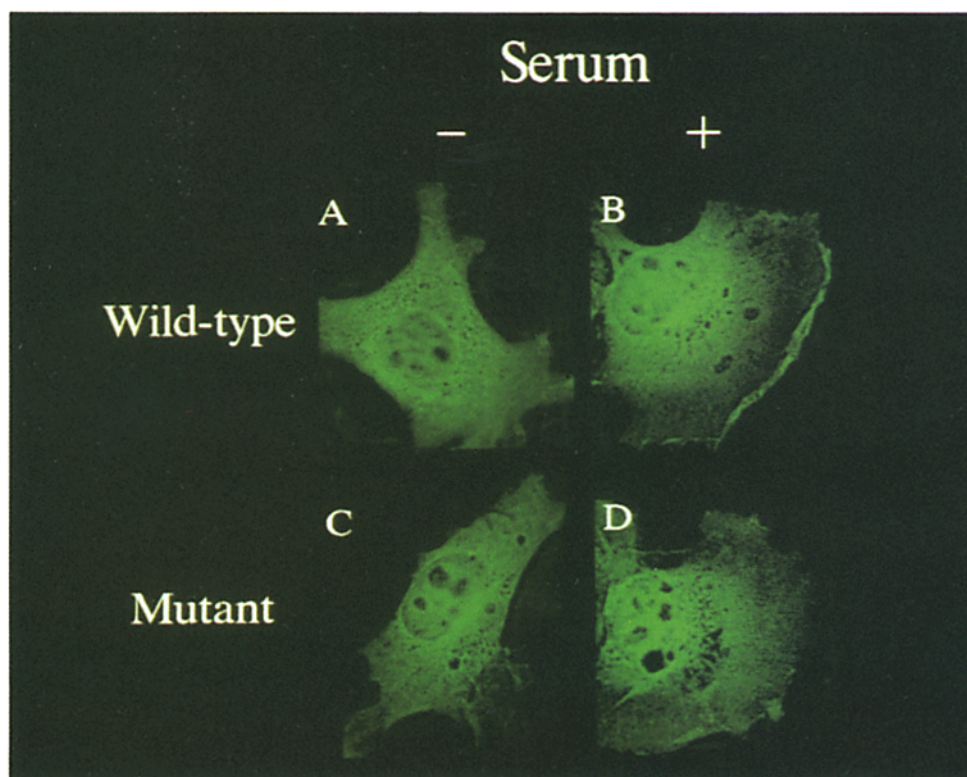
### Discussion

There has been much interest in the role of MAP kinases in signal transduction because these enzymes appear to integrate signaling pathways that are initiated by many types of cell surface receptors (2, 15, 17, 19, 46). Molecular cloning





**Figure 10.** Localization of the ERK2 isoform p40<sup>mapk</sup>. Cells expressing wild-type (A and B) and kinase-negative mutant (C and D) forms of p40<sup>mapk</sup> were serum starved for 18 h and then treated without (A and C) or with (B and D) 10% FBS for 30 min. The cells were fixed and analyzed by indirect immunofluorescence using a mouse monoclonal anti-MAP kinase antibody and an FITC-conjugated goat anti-mouse Ig antibody. All images were obtained by digital imaging microscopy and processed for image restoration. The figure presents representative images of the fluorescence detected from single cells.



**Figure 11.** Localization of the ERK2 isoform p41<sup>mapk</sup>. Cells expressing wild-type (A and B) and kinase-negative (C and D) forms of p41<sup>mapk</sup>. The cells were serum-starved for 18 h and then treated without (A and C) or with (B and D) 10% FBS for 30 min. The cells were then fixed and analyzed by indirect immunofluorescence microscopy using a mouse monoclonal anti-MAP kinase antibody and an FITC-conjugated goat anti-mouse Ig antibody. All images were obtained by digital imaging microscopy. The figure presents representative images of the fluorescence detected from single cells.

of MAP kinases has revealed the presence of isoforms that exhibit heterogeneous expression in human tissues (6, 7, 28). These isoforms have similar *in vitro* substrate specificity (3, 14, 19, 27) and may have similar functions *in vivo*. However, as MAP kinase substrates are located within several cellular compartments, it is possible that these MAP kinase isoforms may have distinct roles during signal transduction because of differences in sub-cellular localization.

We have previously described two isoforms of the human ERK2 protein kinase that are expressed in HeLa cells. These isoforms were designated p40<sup>mapk</sup> and p41<sup>mapk</sup> according to their predicted molecular mass (28). Analysis of the sequence of these protein kinases indicates that they share a high degree of sequence identity except that p41<sup>mapk</sup> has a short NH<sub>2</sub>-terminal Ala-rich extension that is absent in p40<sup>mapk</sup> (Fig. 1). To examine the significance of this difference in structure, we compared the properties of p40<sup>mapk</sup> and p41<sup>mapk</sup>. It was found that these protein kinases exhibited similar chromatographic properties (Figs. 4 and 5) and that both kinases were able to transduce a signal to the nucleus (Fig. 6). Thus, the biochemical properties of p40<sup>mapk</sup> and p41<sup>mapk</sup> are very similar. However, in further studies using immunofluorescence and digital imaging microscopy, it was found that these MAP kinase isoforms differ in their sub-cellular localization. This observation suggests that these MAP kinase isoforms may couple to distinct signal transduction targets in addition to common pathways.

### ***Serum-induced Redistribution of MAP Kinase to the Nucleus***

The nucleus has been demonstrated to be a target of the MAP kinase signal transduction pathway (52). Physiological functions of MAP kinases in the nucleus are likely to include the regulation of gene expression (19). For example, *c-Myc*, *NF-IL6*, *c-Jun*, *TAL1* and *p62<sup>TCF</sup>/Elk-1* have been demonstrated to be phosphorylated by MAP kinases (1, 3, 11, 13, 25, 30, 39, 42, 48, 51, 52). In serum-starved cells only a very low level of MAP kinase is detected in the nucleus. However, treatment of cells with serum causes the redistribution of MAP kinase from the cytosol to the nucleus (3, 10, 49, 52). Previous investigations of the cellular distribution of MAP kinases have been performed without reference to the analysis of individual isoforms (3, 10, 49). Here we show that serum treatment caused the redistribution of two different MAP kinase isoforms (p40<sup>mapk</sup> and p41<sup>mapk</sup>) from the cytosol to the nucleus (Figs. 10 and 11). This serum-stimulated nuclear translocation was also observed for the kinase-negative forms of p40<sup>mapk</sup> and p41<sup>mapk</sup> (Figs. 10 and 11). Together, these data demonstrate that serum causes the nuclear redistribution of both p40<sup>mapk</sup> and p41<sup>mapk</sup> and that intrinsic MAP kinase activity is not required for the nuclear accumulation.

The mechanism of serum-induced translocation of MAP kinases remains unknown because a nuclear localization sequence within the primary structure of p40<sup>mapk</sup> and p41<sup>mapk</sup> has not been identified (28). However, three general mechanisms can be proposed: (a) MAP kinase may enter the nucleus because of a general increase in nuclear transport in serum-treated cells. We are, however, unaware of any precedent for this form of regulation of nuclear localization. (b) It is possible that MAP kinases may have a cryptic nuclear

localization sequence that is exposed by the treatment of cells with serum. Kinase-negative mutant forms of p40<sup>mapk</sup> and p41<sup>mapk</sup> are translocated into the nucleus (Figs. 10 and 11). Thus, intrinsic kinase activation is not required for translocation. However, it is possible that the mechanisms of MAP kinase activation (Thr and Tyr phosphorylation) may act as a signal for translocation. (c) If MAP kinase lacks a functional nuclear localization sequence, it is possible that this signal may be provided by association with another protein. Proteins that could account for this activity have not been identified, but one candidate is represented by MAP kinase kinase (5, 18, 33, 50, 56, 59).

### ***Serum-induced Redistribution of MAP Kinase to the Cell Surface Ruffling Membrane***

The formation of cell surface membrane ruffles is a common event that is observed after the treatment of cells with growth factors. Recent studies have established an important role for small GTP-binding proteins (*rho* and *rac*) in the formation of the membrane ruffles and the reorganization of the cytoskeleton (10, 49). It is possible that the localization of MAP kinase in the membrane ruffles may be related to the regulation of the cytoskeleton in this region of the cell (12, 22, 29). Alternatively, the serum-stimulated redistribution of MAP kinase to the membrane ruffles may function to couple the MAP kinase signal transduction pathway to targets located at the cell surface. Examples of potential targets of MAP kinase at the cell surface (19) include the EGF receptor (16, 44, 53) and phospholipase A<sub>2</sub> (37, 43).

Analysis of the cellular distribution of p41<sup>mapk</sup> demonstrated that this protein kinase is localized at the cell surface ruffling membrane after serum-stimulation (Fig. 11). However, the cell surface translocation of p40<sup>mapk</sup> was not observed (Fig. 10). This observation suggests that the NH<sub>2</sub>-terminal region of p41<sup>mapk</sup> is required for localization at the cell surface (Figs. 10 and 11). Thus, it is possible that the 12-amino acid NH<sub>2</sub>-terminal extension could act as a "signal sequence" for cell surface membrane localization. However, strong evidence against this hypothesis was obtained from the analysis of the kinase-negative MAP kinases which did not localize to the cell surface ruffling membranes after serum treatment. This observation demonstrates that the 12-amino acid NH<sub>2</sub>-terminal extension found in the primary sequence of p41<sup>mapk</sup> is not sufficient to confer serum-dependent localization at the cell surface. Together, these data indicate that the cell surface localization of p41<sup>mapk</sup> in serum-stimulated cells requires both protein kinase activation and the NH<sub>2</sub>-terminal region of p41<sup>mapk</sup>. Further studies are required to identify structures within the cell surface ruffling membrane that are required for the cell surface localization of p41<sup>mapk</sup>.

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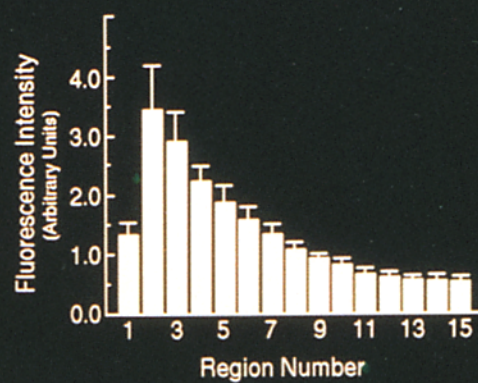
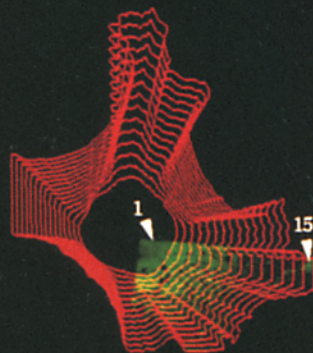
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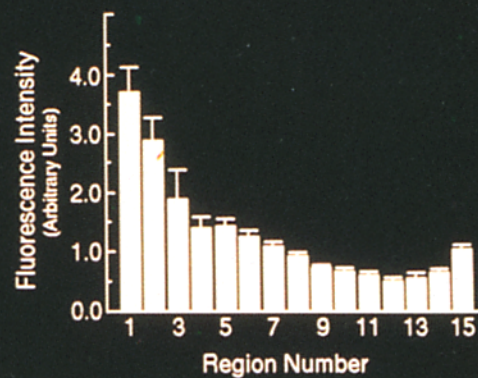
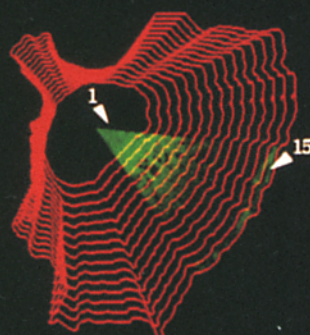


A) p41<sup>mapk</sup> Wild-type

- Serum

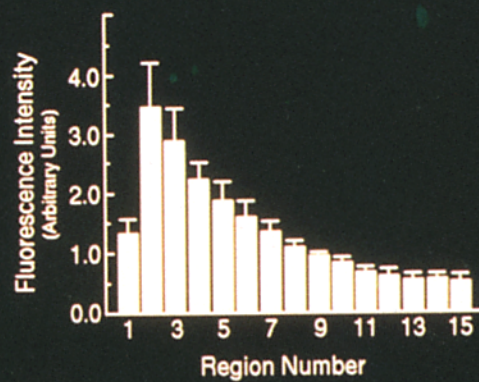
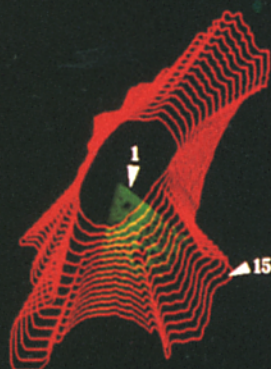


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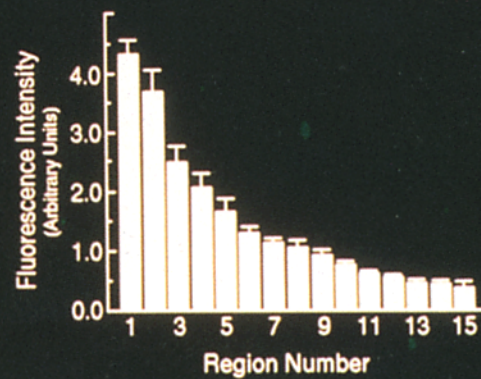
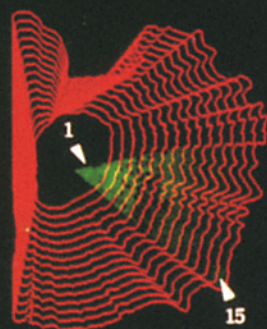


B) p41<sup>mapk</sup> Mutant

- Serum



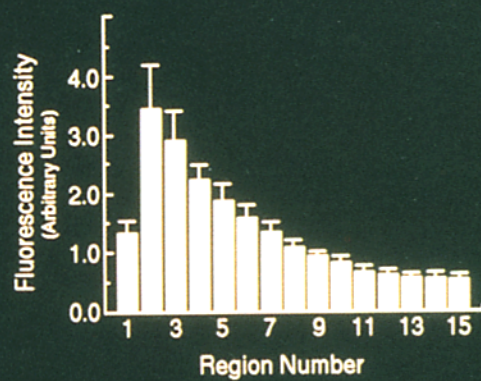
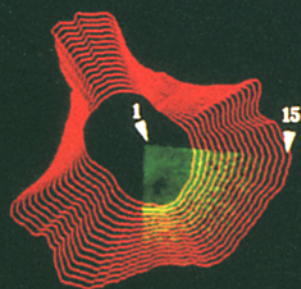
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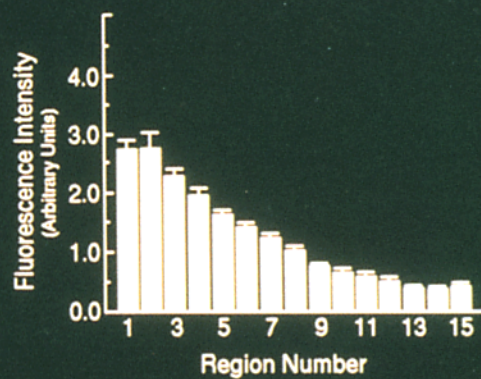
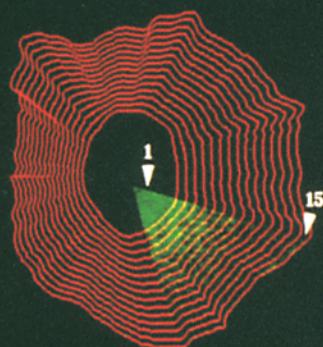


**C) p40<sup>mapk</sup> Wild-type**

- Serum

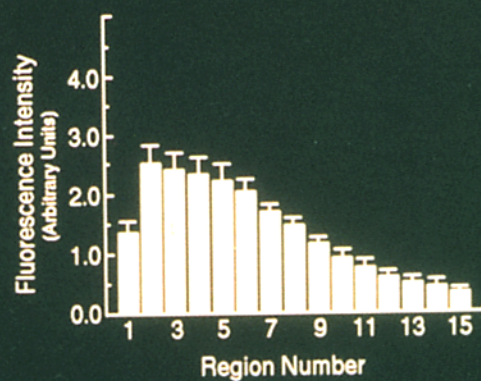
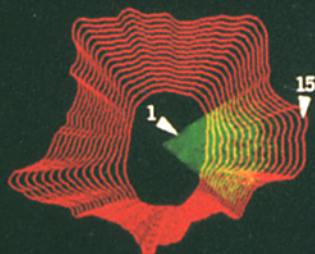


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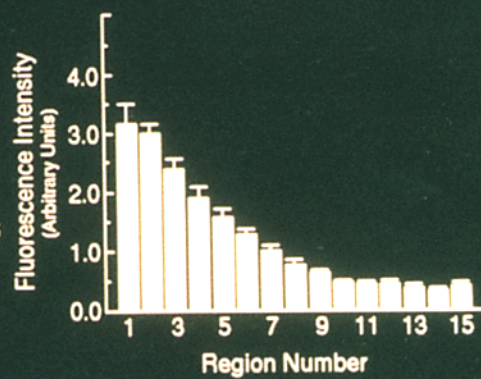
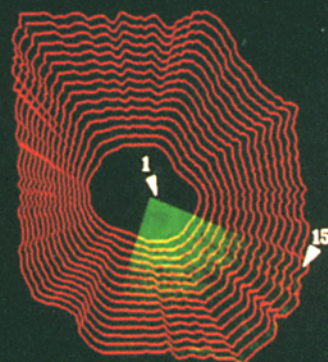


**D) p40<sup>mapk</sup> Mutant**

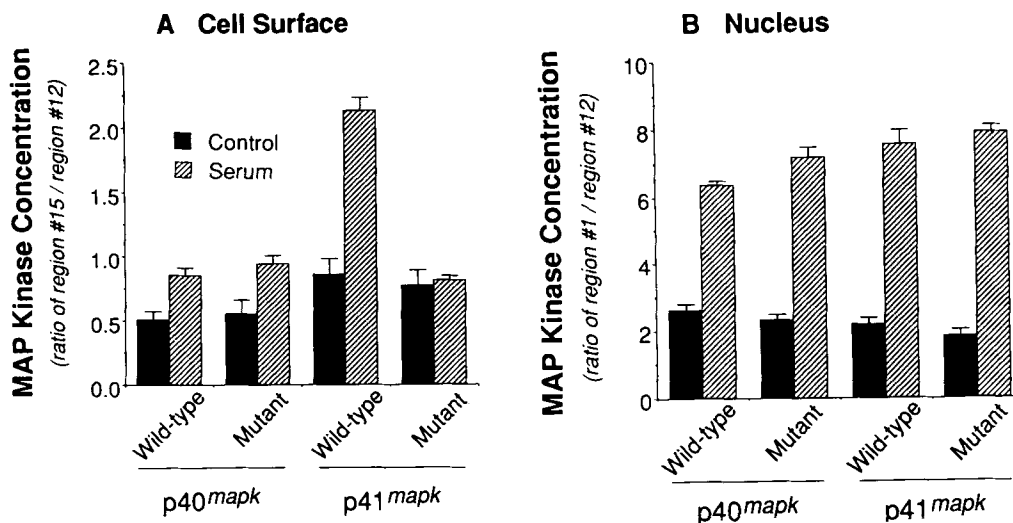
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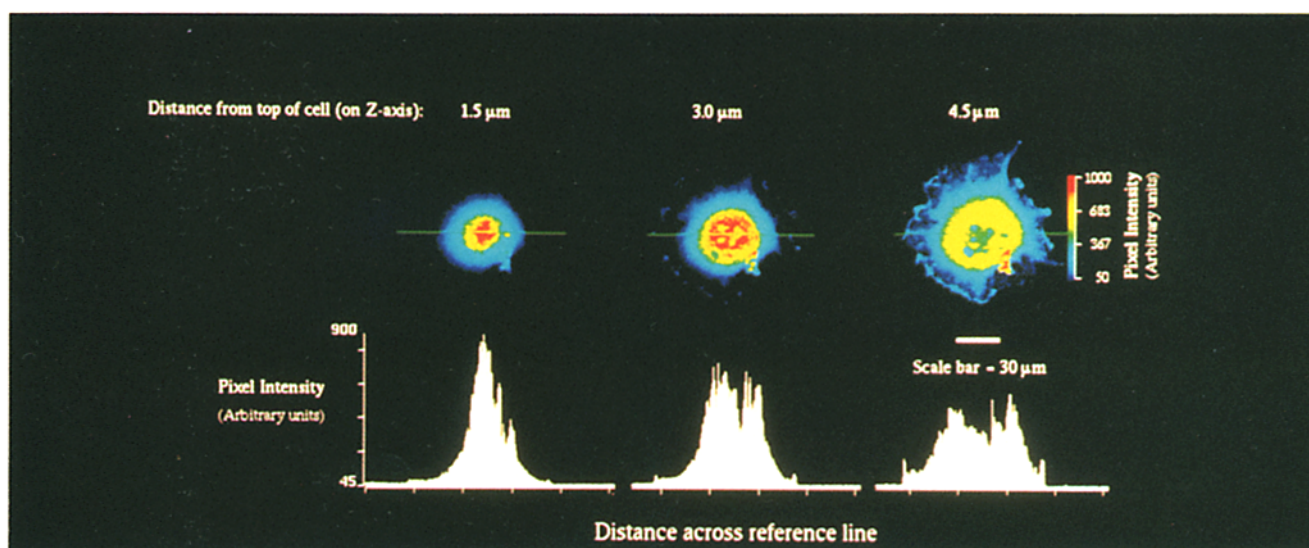
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**Figure 13.** Comparison of MAP kinase distribution in control and serum-treated cells. The relative molecular density of MAP kinase in the nucleus (A) and at the cell surface ruffling membrane (B) is presented (mean  $\pm$  SD,  $n = 8$ ). The density of MAP kinase was normalized by dividing the average pixel intensity (Fig. 10) of region 15 (nucleus) and region 1 (cell surface ruffling membrane) by the average pixel intensity of region 12 (a cytosolic serum-independent region).



**Figure 14.** Analysis of the sub-cellular distribution of p41<sup>mapk</sup> by confocal microscopy. Three different focal planes are shown of a cell incubated with 10% FBS for 30 min. The images shown are representative of cells observed in three separate experiments. The intensity of the fluorescence in each image is shown in pseudocolors and the pixel intensity across the reference line (green) is illustrated. Bar, 30  $\mu$ m.

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**Figure 12.** Quantitative analysis of the cellular distribution of the ERK2 isoforms p41<sup>mapk</sup> and p40<sup>mapk</sup>. The molecular density of p41<sup>mapk</sup> and p40<sup>mapk</sup> was determined from single optical sections that were obtained by digital imaging microscopy, processed for image restoration and quantitated as described in detail under Materials and Methods. The normalized fluorescence intensity in 15 cellular regions are presented as the average  $\pm$  SEM ( $n = 8$ ). The figure presents the quantitation of the distribution of wild-type p41<sup>mapk</sup> (A), kinase-negative p41<sup>mapk</sup> (B), wild-type p40<sup>mapk</sup> (C), and kinase-negative p40<sup>mapk</sup> (D). The effect of the treatment of the cells with 10% FBS for 30 min is presented. Typical cells used in the analysis are illustrated on the left side of each graph. The boundaries of the 15 regions are presented in red and the sector used for analysis is shown in green.

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