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Sequential Activation of ERK and Repression of JNK by Scatter Factor/Hepatocyte Growth Factor in Madin-Darby Canine Kidney Epithelial Cells

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The scattering of Madin-Darby canine kidney (MDCK) epithelial cells by scatter factor/hepatocyte growth factor (SF/HGF) is associated with transcriptional induction of the urokinase gene, which occurs essentially through activation of an EBS/AP1 response element. We have investigated the signal transduction pathways leading to this transcriptional response. We found that SF/HGF induces rapid and sustained phosphorylation of the extracellular signal-regulated kinase (ERK) MAPK while stimulating weakly and then repressing phosphorylation of the JUN N-terminal kinase (JNK) MAPK for several hours. This delayed repression of JNK was preceded by phosphorylation of the MKP2 phosphatase, and both MKP2 induction and JNK dephosphorylation were under the control of MEK, the upstream kinase of ERK. ERK and MKP2 stimulate the EBS/AP1-dependent transcriptional response to SF/HGF, but not JNK, which inhibits this response. We further demonstrated that depending on cell density, the RAS-ERK-MKP2 pathway controls this transrepressing effect of JNK. Together, these data demonstrate that in a sequential manner SF/HGF activates ERK and MKP2, which in turn dephosphorylates JNK. This sequence of events provides a model for efficient cell scattering by SF/HGF at low cell density.

INTRODUCTION

MAPKs are regulated via evolutionarily conserved kinase cascades. In mammalian cells, the best characterized MAPKs are extracellular signal-regulated kinases (ERK1,2), JUN N-terminal kinases (JNK1,2), and p38 MAPK. Most growth factors activate the ERK kinases, primarily through the small GTP-binding protein RAS. Upon stimulation, activated RAS binds the MAPK kinase kinase RAF, which in turn phosphorylates and activates the MAPK kinase MEK1,2 (MKK1,2) and ERK (ERK1,2). This cascade of kinases is often called the RAF-MEK1,2-ERK1,2 kinase module. Similarly, exposure of cells to cytokines and cellular stresses activates the JNK and p38 MAPKs. Through the activation of intermediary kinases, the RHO family small G proteins RAC and CDC42 can regulate the activation of JNK1,2 and p38 MAPKs (Bagrodia *et al.*, 1995; Coso *et al.*, 1995). The proposed corresponding kinase modules are MAPKKK-MKK4,7-JNK1,2 and MAPKKK-MKK3,6-p38 (Su and Karin, 1996; see Dhanasekaran and PremKumar Reddy, 1998). Accumulating evidence also suggests that these kinase modules can be activated by overlapping sets of extracellular stimuli. Indeed, EGF or NGF, through RAS activation and independent of the RAF-MEK-ERK module, can activate JNK in PC12 cells, albeit to a lesser extent than ERK (Minden *et al.*, 1994). Furthermore, some components of these kinase modules can participate in more than one signaling pathway (Fanger *et al.*, 1997). An important goal, therefore, is to understand the mechanisms that allow a single factor to induce specific biological responses while implicating several MAPK modules.

The scatter factor/hepatocyte growth factor (SF/HGF) is a multifunctional growth factor capable of inducing scattering, proliferation, and branching morphogenesis of various epithelial cells (Weidner *et al.*, 1993). The initial event in SF/HGF signaling involves its binding to the c-MET tyrosine kinase receptor. This triggers dimerization and autophosphorylation of the receptor and the recruitment of intracellular proteins (Ponzetto *et al.*, 1994; Weidner *et al.*, 1996), which initiate different signaling pathways. In particular, several lines of evidence suggest that activation of the RAS protein and its downstream effectors, the ERK MAPKs,

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is triggered by several of these proteins and is essential in mediating the biological effects of SF/HGF (Hartmann *et al.*, 1994; Pelicci *et al.*, 1995; Weidner *et al.*, 1996; Nguyen *et al.*, 1997; Potempa and Ridley, 1998; Tanimura *et al.*, 1998; Tulasne *et al.*, 1999). Therefore, like other factors that bind tyrosine kinase receptors, SF/HGF is capable of activating a RAS-RAF-MEK-ERK pathway that promotes biological responses to this factor. Recently, it was also found that JNK is activated by SF/HGF in primary cultures of rat hepatocytes and that it plays a role in mediating their proliferation (Auer *et al.*, 1998). In a human keratinocyte cell line, JNK was also found to be activated by SF/HGF, but it was demonstrated that sustained activation of ERK by SF/HGF is involved in matrix metalloproteinase-9 induction and colony dispersion (McCawley *et al.*, 1999). In addition, it was found that the oncogenic form of c-MET, TPR-MET, activates JNK in FR3T3 fibroblast cells, an activation that seemed to be required for their transformation (Rodrigues *et al.*, 1997). These data suggest that SF/HGF can activate distinct MAPKs that appear to be involved in various biological responses to SF/HGF.

We previously demonstrated that SF/HGF induces transcriptional activation of the urokinase plasminogen activator (uPA) and collagenase gene promoters (Fafeur *et al.*, 1997). These matrix-degrading enzymes belong to complex enzyme cascades that catalyze degradation of extracellular matrix components and facilitate cell scattering. In agreement with this, increased expression of these genes correlates with progressive cell scattering induced by SF/HGF in Madin-Darby canine kidney (MDCK) epithelial cells (Pepper *et al.*, 1992; Fafeur *et al.*, 1997). These transcriptional activations implicate a functional EBS/AP1 response element and led us to further demonstrate that the scattering signal of SF/HGF involves activation of the RAS-ERK pathway (Tulasne *et al.*, 1999). In the present study, we examined whether the MAPK JNK is also implicated in transmitting SF/HGF action.

MATERIALS AND METHODS

Cell Culture and Reagents

MDCK epithelial cells (kindly provided by Dr. Jacqueline Jouanneau, Ecole Nationale Supérieure, Paris) were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FCS and antibiotics at 37°C. Human recombinant forms of SF/HGF and tumor necrosis factor-α (TNF-α) were purchased from R&D Systems (Minneapolis, MN). The MEK inhibitor U0126 was purchased from Promega (Madison, WI). Okadaic acid and sodium vanadate were purchased from Sigma Chemical (St. Louis, MO). Pervanadate treatment of the cells was performed by incubating the cells in DMEM at 37°C with a 10 mM H_2O_2, 3 μM sodium vanadate solution (Beauchemin *et al.*, 1997). Calf intestine alkaline phosphatase was purchased from Boehringer Mannheim (Indianapolis, IN).

Antibodies

Anti-phospho-ERK1 (anti-ACTIVE MAPK) rabbit polyclonal antibody was purchased from Promega, and anti-phospho-p38 MAPK (Thr 180/Tyr 182) rabbit polyclonal antibody was purchased from New England Biolabs (Beverly, MA). Anti-phospho-JNK (anti-ACTIVE JNK) rabbit polyclonal antibody purchased from Promega and anti-phospho-JNK rabbit polyclonal antibody purchased from New England Biolabs gave similar results. According to the manufacturers' indications, these antibodies were developed with the use of synthetic peptides phosphorylated on both the Thr and Tyr residues within the enzyme's catalytic core. The rather high conservation of these sequences between ERK and JNK led us to further identify the phosphorylated form of these kinases be means of rehybridization with their respective antibodies. Anti-ERK1 (C16), anti-JNK1 (C17), and anti-p38 (C20) rabbit polyclonal antibodies and anti-MKP1 (C19) and anti-MKP2 (S18) affinity-purified rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids

The pcDNA-neo expression vector encoding ERK1 (ERK1) was provided by Dr. Philippe Lenorman (Centre National de la Recherche Scientifique, Unité Mixte de Recherche 6543, Nice, France). The pcDNA3 encoding FLAG-tagged wild-type JNK1 (JNK1) and inactivated JNK1 ($JNK1^{APF}$) were provided by Dr. Benoit Dérijard (Centre National de la Recherche Scientifique, Unité Mixte de Recherche 6543, Nice, France). The prcCMV encoding inactivated ERK1 ($ERK1^{TA}$) was provided by Dr. Pascale Crepieux (Institut National de la Recherche Agronomique, Tours, France). The pEXV3 encoding inactivated RAS (RAS^{S186}) was provided by Dr. Stéphane Ansieau (Max Delbruck Centrum, Berlin). The pMT90 encoding inactivated CDC42 ($CDC42^{N17}$) was provided by Dr. Philippe Chavrier (Center d'Immunologie, Marseille, France). The pSG5 expression vector encoding MKP1 (MKP1) and the pcDNA-neo expression vector encoding MKP2 (MKP2) were provided by Dr. Jacques Pouysségur (Centre National de la Recherche Scientifique, Unité Mixte de Recherche 6545, Nice, France). The GST-JUN^{1-79} bacterial vector was provided by Dr. Benoit Dérijard. The EBS/AP1-Luc reporter contains three tandem copies of a polyoma virus enhancer–derived sequence with EBS/AP1-binding sites linked to the thymidine kinase promoter and drives the luciferase reporter gene (Fafeur *et al.*, 1997).

Transactivation Assays

The transactivation assays were performed essentially as described previously (Fafeur *et al.*, 1997). MDCK cells (30,000) were cultured on 12-well plates for 1 d and then transiently transfected with the use of a lipofection method. Cells were rinsed and incubated in 500 μl of Opti-MEM (Life Technologies-BRL) with a mixture of DNA (2.5 μg/ml) and Lipofectamine (20 μg/ml) (Life Technologies-BRL). In each experiment, cells were incubated with the same total amount of plasmid DNA, completed as necessary with the corresponding empty expression vector. After 6 h, 500 μl of Opti-MEM containing 20% FCS was added, and cells were incubated overnight. The cells were then rinsed and incubated in DMEM–0.5% FCS in the presence or absence of SF/HGF (10 ng/ml). Twenty-four hours later, cells were disrupted in reporter lysis buffer (Promega) and assayed for protein content and luciferase activity. Fold activation is the ratio from each luciferase value relative to the value from the reporter gene with empty expression vector. Each experiment was repeated at least twice with independent plasmid preparations to assess reproducibility. In parallel, we repeatedly checked for efficiency of transfection with the use of a pGFP plasmid, which was routinely 40–60%.

Immunoblot

MDCK cells (400,000 cells per 100-mm dish) were cultured for 1 d in DMEM–10% FCS. The next day, cells were incubated in DMEM–0.5% FCS for an additional 24 h and then treated with distinct reagents for the times indicated on the Figures. After treatment, cells were washed twice with PBS and suspended in lysis buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 0.25 mM EDTA, 0.1% NP40, 10 mM NaF) containing freshly added protease and phosphatase inhibitors (20 mM β-glycerophosphate, 1

mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Lysates were clarified by centrifugation at 4°C, and protein concentration was determined by Bio-Rad (Richmond, CA) protein assay. Cell lysates (20–30 μg) for direct analysis by immunoblotting were resuspended in Laemmli sample buffer, boiled for 5 min, and separated onto 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride filters (Millipore, Bedford, MA). The filters were incubated with blocking buffer (0.2% [vol/vol] casein, 0.1% [vol/vol] Tween-20 dissolved in PBS) for 1 h at room temperature and probed for 1 h at room temperature with appropriate antibodies diluted in blocking buffer according to the manufacturer's recommendations. After extensive washing in PBS/Tween 0.2%, immune complexes were detected with species-specific secondary antiserum conjugated with alkaline phosphatase followed by an enhanced chemiluminescence detection system (Aurora, ICN Biomedical, Costa Mesa, CA). For membrane reprobing, filters were incubated for 30 min at 55°C in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7). After extensive washing, filters were incubated in blocking buffer and reprobed with specific antibodies.

Phosphatase Assay

MDCK cells (400,000 cells per 100-mm dish) were cultured for 1 d in DMEM–10% FCS. The next day, cells were incubated in DMEM–0.5% FCS for an additional 24 h and then treated with SF/HGF for 10 min. Cell extracts were immunoprecipitated with the anti-MKP2 antibody as described below and then incubated for 1 h at 37°C with or without 20 U of alkaline phosphatase in 100 μl of phosphatase buffer (50 mM Tris, pH 8, 100 mM NaCl, 5 mM $MgCl_2$). Phosphatase reactions were stopped by the addition of Laemmli sample buffer, and MKP2 detection was performed by immunoblotting as described above.

JNK Activity Assay

MDCK cells (400,000 cells per 100-mm dish) were cultured for 1 d in DMEM–10% FCS and then transiently transfected with JNK1 with the use of the lipofection method described above. The next day, cells were grown in serum-free minimal Eagle's medium (Life Technologies). Twenty-four hours later, cells were stimulated for 10 min with 30 ng/ml SF/HGF. After treatment, cells were washed twice with PBS and suspended in lysis buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 0.25 mM EDTA, 0.1% NP40, 10 mM NaF) containing freshly added protease and phosphatase inhibitors (20 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Lysates were clarified by centrifugation at 4°C, and protein concentration was determined by Bio-Rad protein assay. The supernatant was incubated with an anti-FLAG antibody (M2, Sigma) overnight at 4°C and then incubated with protein A–Sepharose beads (Pharmacia, Piscataway, NJ) for 1 h at 4°C. Immune complexes were washed four times with ice-cold lysis buffer and once with kinase buffer (20 mM MOPS, pH 7.2, 7.5 mM $MgCl_2$, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na_3VO_4, 1 mM DTT). The kinase reaction was carried out for 30 min at 30°C in the presence of GST-JUN (3 μg), 50 μM ATP, and 10 μCi of [γ-^{33}P]ATP (2000 Ci/mmol) (ICN). The phosphorylated JUN protein was resolved by 10% SDS-PAGE and dried, and incorporation of [γ-^{33}P]ATP into substrate protein was quantified with the use of a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and visualized onto hyperfilms-MP (Amersham, Arlington Heights, IL) by autoradiography.

Cell-scattering Assay

Scattering from cell islets was assayed as follows: MDCK cells were seeded onto 12-well plates and cultured for 2–3 d until they formed colonies. Then they were cultured for 24 h, with or without 10 ng/ml SF/HGF in DMEM–0.5% FCS. At the end of the experiments, cells were fixed and stained (Diff-Quik, Dade AG, Düdingen, Switzerland) and their morphology was examined by light microscopy.

RESULTS

SF/HGF Induces Differently the Phosphorylation of ERK and JNK

Since their original identification, it has been known that activation of either ERK or JNK is mediated by dual phosphorylation on tyrosine and threonine residues (Cano and Mahadevan, 1995). In various cell types, including MDCK epithelial cells, SF/HGF was found to induce phosphorylation of ERK (Potempa and Ridley, 1998; Tanimura *et al.*, 1998; Tulasne *et al.*, 1999). In other cell types, it was also found that SF/HGF can induce transient phosphorylation of JNK (Auer *et al.*, 1998; McCawley *et al.*, 1999) and p38 (McCawley *et al.*, 1999). To investigate whether SF/HGF can regulate the phosphorylation of ERK, JNK, and p38 in canine MDCK epithelial cells, cellular extracts were prepared after stimulation by SF/HGF and immunoblotted with the use of anti-phospho-MAPKs (Figure 1). Their identification was obtained by reprobing the blots with their respective anti-MAPK antibodies. This was particularly important in that all of these kinases and their phosphorylated forms migrate within a range of 40–50 kDa. It is noteworthy that the anti-phospho-JNK kinase antibody recognized both the phosphorylated forms of JNK (arrow) and ERK (open arrowhead), which were distinguishable with the use of well-separated 10% SDS gels (Figure 1, middle panel). JNK activity assays were also performed with the use of GST-JUN as a substrate.

As shown in Figure 1A, within 10 min, SF/HGF induced strong phosphorylation of ERK1,2, whereas it induced weak phosphorylation of JNK1 and had no effect on p38. We compared these effects of SF/HGF with those of TNF-α, a known activator of JNK in various cell types. In contrast to SF/HGF, TNF-α induced weak phosphorylation of ERK1,2 and robust phosphorylation of JNK1 and p38 (Figure 1B). The phosphorylation of JNK2 was also found to be induced weakly by SF/HGF and strongly by TNF-α (our unpublished results). The results regarding JNK phosphorylation were confirmed by measuring JNK kinase activity. After transfection of wild-type JNK1, the kinase was immunoprecipitated and tested for its ability to phosphorylate GST-JUN (Derijard *et al.*, 1994). The activity of JNK was induced twofold by SF/HGF and fivefold by TNF-α (Figure 1). These results show that SF/HGF is a potent activator of ERK but not of JNK, whereas TNF-α is a potent activator of JNK and p38 but not of ERK.

We also determined the long-term effects of SF/HGF on the phosphorylation of these kinases. SF/HGF induced strong and sustained phosphorylation of ERK for several hours (Figure 2A). In contrast, within a few hours SF/HGF induced a sustained repression of JNK: the amount of phosphorylated JNK was lower than in the control after 4 h of treatment by SF/HGF and remained below the basal level for 8 h (Figure 2A). In subsequent experiments, we found that dephosphorylation was already detectable after 2 h of treatment by SF/HGF (see Figures 3 and 8).

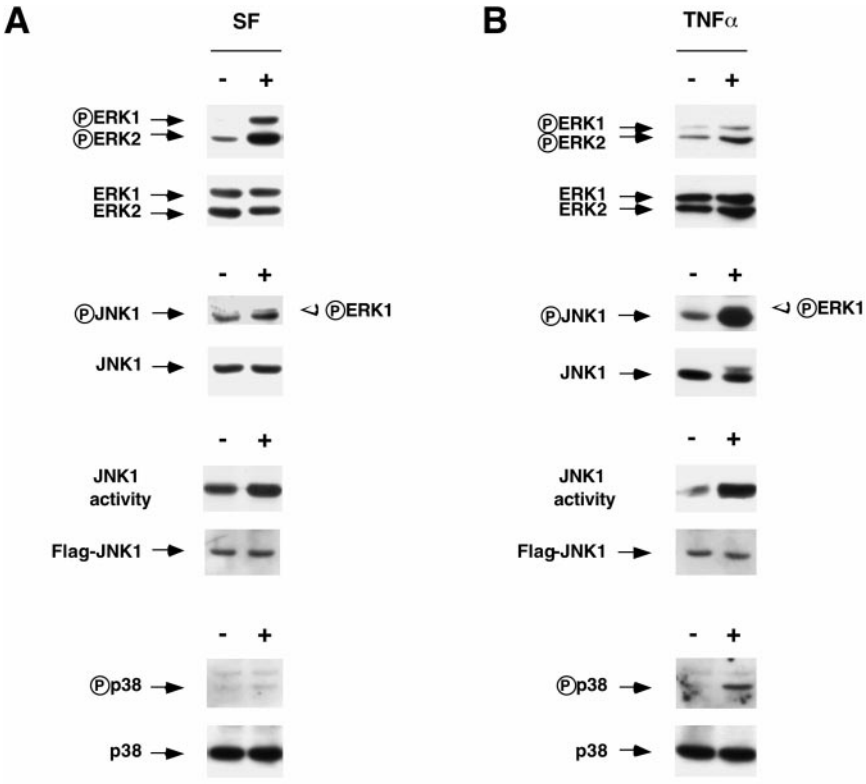

Figure 1. Short-term effects of SF/HGF and TNF-α on phosphorylation of ERK, JNK, and p38 MAPKs. (A) Cells were treated for 10 min with or without 30 ng/ml SF/HGF. Whole cell lysates were collected, and 20 μg of cell extracts was fractionated by 10% SDS-PAGE and examined by immunoblot analysis with the use of either anti-phospho-MAPK antibodies or their respective anti-MAPK antibodies. Positions of ERK1,2, JNK1, and p38 are shown by arrows, and their phosphorylated forms are indicated by a circled P. JNK1 activity was evaluated by a specific immunoprecipitation/kinase assay with the use of GST-JUN$^{1-79}$ as a substrate; immunoblot of FLAG-tagged JNK1 with anti-FLAG antibody indicates comparable loading. (B) Cells were treated for 10 min with or without 30 ng/ml TNF-α. Cell extracts and immunoblot analysis were performed as described above. The filters used for examination of phospho-MAPKs were stripped and reprobed with their respective anti-MAPK antibodies. Symbols are as in A. It is worth noting that the anti-phospho-JNK kinase antibody recognized the phosphorylated forms of both JNK1 (left arrow) and ERK1 (right open arrowhead).

The phosphorylation of p38 was not modified by SF/HGF, even after several hours of treatment (our unpublished results).

The dephosphorylation of JNK occurred in a concentration-dependent manner. After 4 h of SF/HGF treatment, both ERK phosphorylation and JNK dephosphorylation increased, with increasing concentrations of SF/HGF ranging from 0.1 to 30 ng/ml (Figure 2B). The effective concentrations of SF/HGF (>1 ng/ml) correspond to those obtained in a cell-scattering assay (our unpublished results).

These results show that SF/HGF induces a rapid and prolonged phosphorylation of ERK for several hours, whereas it induces a rapid and weak phosphorylation of JNK, which was followed within a few hours by a sustained repression of JNK phosphorylation.

SF/HGF Induces Expression of the MKP2 Phosphatase

To determine whether dephosphorylation of JNK can be caused by the activation of a phosphatase, MDCK cells were pretreated with protein phosphatase inhibitors, pervanadate, and okadaic acid, which are known to inhibit the tyrosine phosphatases and type 1–type 2A serine–threonine phosphatases, respectively (Guo *et al.*, 1998). As shown in Figure 3, SF/HGF induced dephosphorylation of JNK within 2 h. In the absence of SF/HGF, treatment with okadaic acid (Figure 3A) or pervanadate (Figure 3B) resulted in an increase in the amount of phosphorylated JNK. In the presence of SF/HGF, pervanadate, but not okadaic acid, prevented the dephosphorylation of JNK (Figure 3, A and B). These results indicate that dephosphorylation of JNK by SF/HGF occurs mainly by a pervanadate-sensitive tyrosine phosphatase.

The tyrosine phosphatases of the MAPK phosphatase (MKP) family, such as MKP1 and MKP2, which exhibit dual catalytic activity toward phosphotyrosine and phosphothreonine, are of special interest in the regulation of intracellular MAPK signaling pathways (Keyse and Emslie, 1992; King *et al.*, 1995; Misra-Press *et al.*, 1995; Camps *et al.*, 2000; Keyse, 2000). To examine their possible involvement in the modulation of JNK phosphorylation, we performed immunoblotting experiments with the use of cellular extracts obtained in the previous kinetic and dose–response experiments (Figure 2) and antibodies against the phosphatases MKP1 and MKP2. SF/HGF induced the expression of both MKP2 and MKP1 within 1 h (Figure 4, A and B). MKP1 was not easily detected in MDCK canine cells. We do not know whether this is because the amount of MKP1 is low in MDCK canine cells or because the endogenous MKP1 phosphatase is not well recognized by an antibody raised against the human form of MKP1. In contrast, both expression and phosphorylation of MKP2 were clearly induced by SF/HGF within 10 min. Indeed, treatment with alkaline phosphatase demonstrated that the slowly migrating band (Figure 4, A and B) is a phosphorylated form of MKP2 (Figure 4C). This time course is consistent with MKP2 phosphorylation and expression being induced before the dephosphorylation of JNK (1–4 h; Figure 2A, lower panel), which occurred before

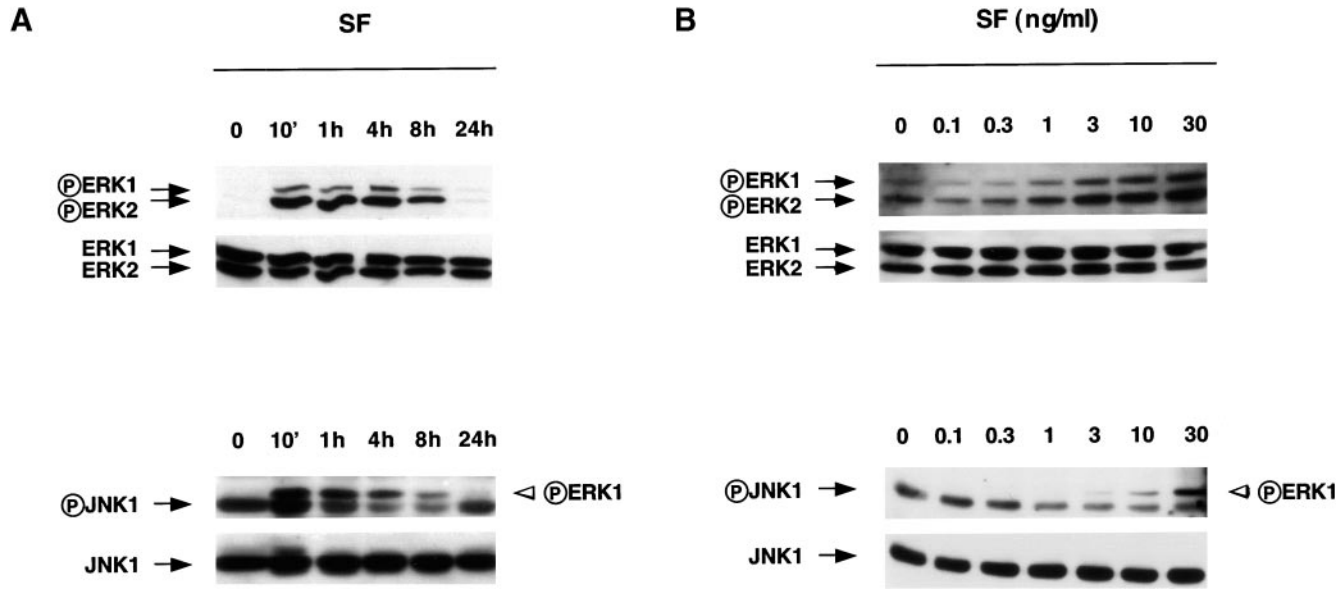

Figure 2. Long-term effects of SF/HGF on phosphorylation of ERK and JNK MAPKs. (A) Cells were treated for the indicated times with SF/HGF (30 ng/ml). Cell extracts (20 μg for ERK determination, 30 μg for JNK determination) were fractionated on 10% SDS-PAGE. Immunoblot analysis was performed with the use of the anti-phospho-ERK kinase antibody (top panel) or the anti-phospho-JNK kinase antibody (bottom panel). The filters used for examination of phospho-MAPKs were stripped and reprobed with their respective anti-MAPK antibodies. Positions of ERK1,2 and JNK1 are shown by arrows, and their phosphorylated forms are indicated by a circled P. Middle panel, the anti-phospho-JNK kinase antibody recognized the phosphorylated forms of both JNK1 (left arrow) and ERK1 (right open arrowhead). (B) Cells were treated for 4 h with increasing concentrations of SF/HGF (0.1–30 ng/ml). As described for A, cell extracts and immunoblot analysis were performed with the use of the anti-phospho-ERK kinase antibody (top panel) or the anti-phospho-JNK kinase antibody (bottom panel).

Figure 3. Effects of okadaic acid and pervanadate on SF/HGF-induced JNK dephosphorylation. Cells were pretreated for 30 min with 250 nM okadaic acid (OA; A) or 3 μM pervanadate (PerV; B) and were then treated for 2 h with or without SF/HGF (30 ng/ml). Cell extracts, immunoblot analysis of phosphorylated JNK1, and rehybridization with the anti-JNK1 antibody were performed as described in Figure 2. The band corresponding to JNK1 and its phosphorylated form is large and was also found with the use of a lower exposure of the gel because the 10% SDS-PAGE was particularly well separated.

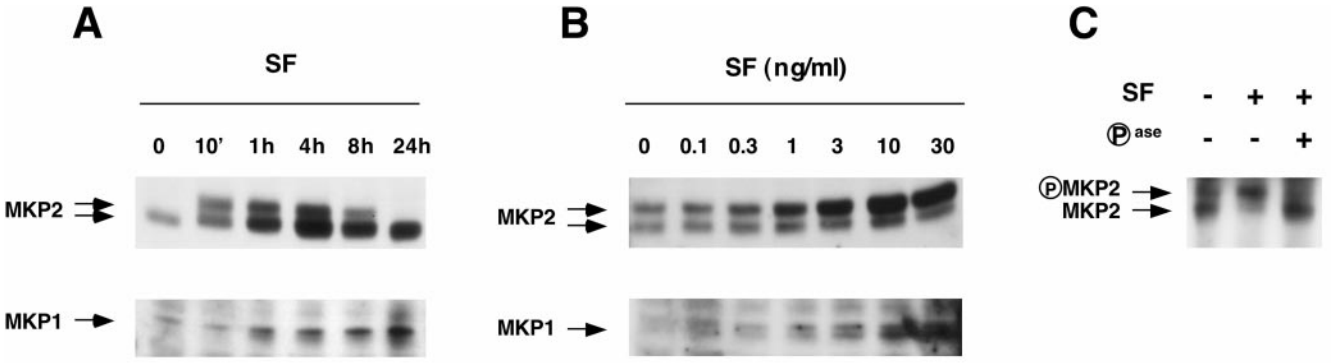

Figure 4. Long-term effects of SF/HGF on expression of MKP1 and MKP2 phosphatases. (A) Cells were treated for the indicated times with SF/HGF (30 ng/ml). Cell extracts (30 μg) were fractionated by 10% SDS-PAGE, and immunoblot analysis was performed with the use of the anti-MKP2 or anti-MKP1 antibody. MKP1 and MKP2 migrated at their expected sizes (~42 and 39 kDa, respectively). (B) Cells were treated for 4 h with increasing concentrations of SF/HGF (0.1–30 ng/ml). Cell extracts (30 μg) were fractionated by 10% SDS-PAGE, and immunoblot analysis was performed with the use of the anti-MKP2 or anti-MKP1 antibody. (C) Cells were treated for 10 min with (+) or without (−) SF/HGF (30 ng/ml). Immunoprecipitated MKP2 was incubated (+) or not (−) with alkaline phosphatase (Pase), and MKP2 detection was performed by immunoblot analysis with the anti-MKP2 antibody.

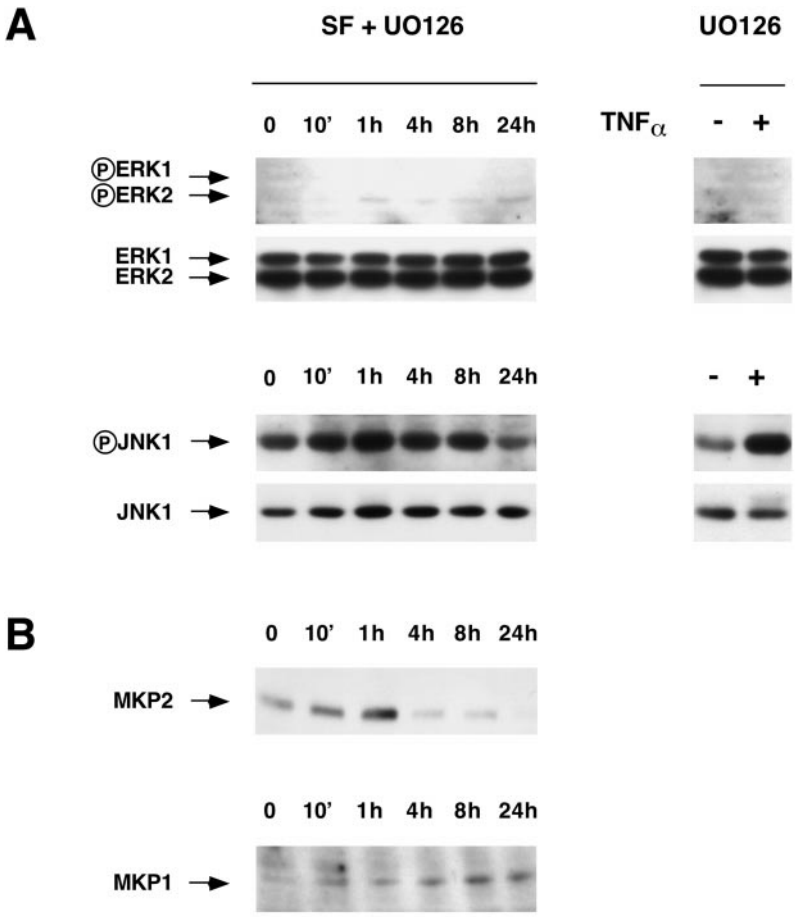

Figure 5. Effects of U0126 on MAPKs and MKP phosphatases induced by SF/HGF. (A) Cells were pretreated with U0126 (25 μM) for 30 min and then treated for the indicated times with 30 ng/ml SF/HGF (SF + U0126) or for 10 min with or without 30 ng/ml TNF-α (right panel). Cell extracts and immunoblot analysis were performed as described in Figure 2 with the use of the anti-phospho-ERK kinase antibody (top panel) or the anti-phospho-JNK kinase antibody (bottom panel). The filters used for examination of phospho-MAPKs were stripped and reprobed with their respective anti-MAPK antibodies. (B) Cell extracts obtained in A were analyzed by immunoblot with the use of the anti-MKP2 or anti-MKP1 antibody.

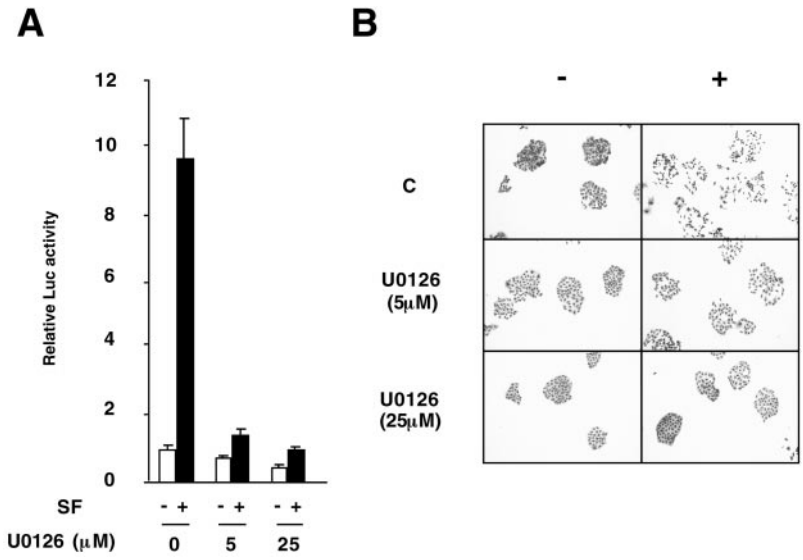

Figure 6. Effects of U0126 on EBS/AP1-dependent transactivation and cell scattering induced by SF/HGF. (A) Cells were seeded on 12-well plates (30,000) and were transfected with the EBS/AP1-Luc reporter vector. The next day, cells were pretreated with U0126 (5 or 25 μM) for 30 min and then left untreated (white bars) or treated with SF/HGF (10 ng/ml) (black bars), and luciferase activity was measured 24 h later. (B) Cells were seeded on 12-well plates (2500) until they formed colonies. Cells were then treated with U0126 (5 or 25 μM) for 30 min. After this time, SF/HGF (10 ng/ml) was added and the cells were further cultured for 24 h.

the dephosphorylation of ERK (4–8 h; Figure 2A, upper panel). These inductions of MKP2 and MKP1 also occurred in a concentration-dependent manner (Figure 4B), with SF/HGF effective concentrations corresponding to those regulating ERK or JNK phosphorylation at 4 h (Figure 2).

Inhibition of MEK Impairs ERK and MKP2 Induction, Restores JNK Phosphorylation, and Impairs RAS-dependent Transcriptional Response and Cell Scattering Induced by SF/HGF

We then investigated whether the sustained activation of ERK and the delayed repression of JNK occurred as a consequence of the activation of the RAS-MEK pathway. To test this hypothesis, we used U0126, a pharmacological inhibitor of the MAPK kinase MEK, which prevents ERK phosphorylation (Favata *et al.*, 1998). As expected, U0126 inhibited the phosphorylation of ERK induced by SF/HGF or TNF-α at all times examined (compare Figures 1, 2A, and 5A). This effect is specific, because U0126 did not affect the rapid induction of JNK phosphorylation induced by TNF-α (compare Figures 1B and 5A). This inhibitor also prevented the induction of MKP2 phosphorylation and expression by SF/HGF, and MKP2 expression level was below the basal level within 24 h (compare Figures 4A and 5B). In contrast, MKP1 expression was still induced within 4 h, and the same result was obtained in the absence of SF/HGF (our unpublished results). Finally, the delayed repression of JNK phosphorylation was no longer observed (compare Figures 2B and 5A). These results demonstrated that inhibition of the MEK kinase also prevented SF/HGF from inducing phosphorylation and expression of MKP2 and dephosphorylation of JNK.

As reported previously, we identified specific transcriptional responses to SF/HGF involving an EBS/AP1 response element, which is the target of a RAS-dependent signal transduction pathway (Fafeur *et al.*, 1997; Tulasne *et al.*, 1999). The effect of the pharmacological inhibition of MEK on the activation of this transcriptional response and on cell scattering was also examined. Transactivation assays were performed by transfecting the cells with the EBS/AP1-Luc reporter vector, which contains three tandem copies of EBS/AP1 binding sites (Tulasne *et al.*, 1999). After transfection, the cells were treated with U0126 and/or SF/HGF for 24 h, at which time transactivation assays were performed. The transcriptional response of the EBS/AP1 response element to SF/HGF was inhibited by U0126 (Figure 6A). Similar results were obtained with the use of a uPA-Luc promoter (our unpublished results), which contains functional EBS/AP1 binding sites (Rorth *et al.*, 1990). For the scattering assay, the cells were seeded sparsely and were grown until they formed colonies. At that time, the cells were treated with U0126 and/or SF/HGF. Within 24 h, cell scattering induced by SF/HGF was impaired in cells treated with U0126 (Figure 6B). These results demonstrated that EBS/AP1-dependent transcriptional responses and cell scattering are also caused by activation of the RAS-MEK pathway.

Transrepression by JNK at High Cell Density

Finally, we wished to investigate the roles of ERK and JNK in transmitting an EBS/AP1-dependent transcriptional response to SF/HGF. During initial experiments, a lack of reproducibility of some transactivation results, in particular when we tested the effects of JNK, led us to identify the influence of cell density. Overall, it was our common observation that cells seeded at low density respond better to the scattering signal of SF/HGF than cells seeded at high density. For example, all cell islets were dissociated by SF/HGF at low cell density (Figure 7A) but not at higher cell density (Figure 7B). We also observed that the amplitude of the EBS/AP1-dependent transcriptional response to SF/HGF was higher at low cell density. For example, activation by SF/HGF was 28-fold for the lowest cell density tested (Figure 7C), whereas it was 12-fold for the higher cell density (Figure 7D). It is worth noting that the highest density tested corresponds to a confluence of 40–50%, which is the usual condition for cell transfection, and to the condition used in all previous experiments. In parallel experiments, we mea-

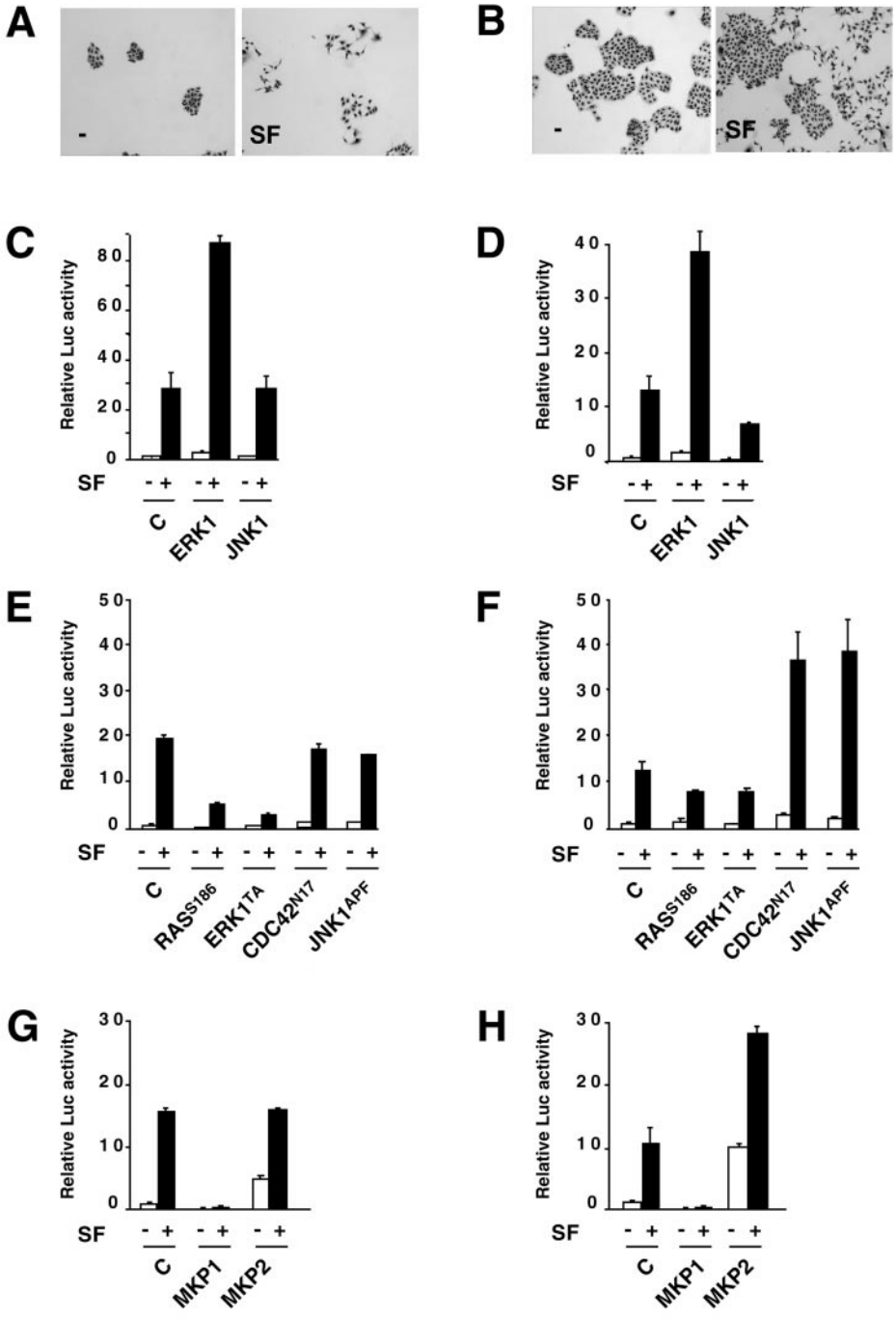

Figure 7. Effects of ERK1, JNK1, MKP2, and MKP1 in inducing an EBS/AP1-dependent transcriptional response at different cell densities. (A and B) Scattering. Cells were seeded on 12-well plates (1250 cells [A] and 5000 cells [B]) and cultured for 2 d. The medium was replaced by DMEM–0.5% FCS, and SF/HGF (10 ng/ml) was added (SF) or not (−) for 24 h. (C and D) Effects of ERK1 and JNK1 on SF/HGF-induced transactivation of the EBS/AP1-Luc reporter vector. Cells were seeded on 12-well plates (10,000 cells [C] and 30,000 cells [D]). The next day, they were cotransfected with the EBS/AP1-Luc reporter vector and with expression vectors, either empty (C) or encoding wild-type ERK1 (ERK1) or wild-type JNK1 (JNK1). The following day, cells were left untreated (−) or treated with 10 ng/ml SF/HGF (+), and luciferase activity was measured 24 h later. The usual condition of transfection is obtained by seeding the cells at 30,000 cells per well, which gives a confluence of 50–60% at the end of the experiment. It is worth noting that at the end of the assay, the sizes of the cell islets are comparable between the cell-scattering (A and B) and transactivation (C and D) assays because of cell mortality during the transfection procedure. (E and F) Effects of dominant negative mutants of RAS, ERK1, CDC42, and JNK1 on SF/HGF-induced transactivation of the EBS/AP1-Luc reporter vector. Cells were seeded on 12-well plates (10,000 cells [E] and 30,000 cells [F]). The next day, they were cotransfected with the EBS/AP1-Luc reporter vector and with expression vectors, either empty (C) or encoding dominant negative forms of RAS (RAS^{S186}), ERK ($ERK1^{TA}$), CDC42 ($CDC42^{N17}$), or JNK ($JNK1^{APF}$). The following day, cells were treated with SF/HGF (10 ng/ml) (black bars) or not (white bars), and luciferase activity was measured 24 h later. (G and H) Effects of MKP1 and MKP2 on SF/HGF-induced transactivation of the EBS/AP1-Luc reporter vector. Cells were seeded on 12-well plates (10,000 cells [G] and 30,000 cells [H]). The next day, they were cotransfected with the EBS/AP1-Luc reporter vector and with expression vectors, either empty (C) or encoding wild-type MKP1 (MKP1) or MKP2 (MKP2). The following day, cells were treated with SF/HGF (10 ng/ml) (black bars) or not (white bars), and luciferase activity was measured 24 h later.

sured transfection efficiencies with the use of cells transfected with a pGFP reporter vector and FACS analysis of the GFP fluorescent cells. The number of seeded cells on 12-well plates (12,500, 25,000, 50,000, and 100,000 cells) did not modify the transfection efficiency, which was 53, 46, 45, and 48%, respectively.

To compare the effects of MAPKs on the transcriptional response of SF/HGF, we cotransfected wild-type ERK1 or wild-type JNK1 and the EBS/AP1-Luc reporter vector (Figure 7, C and D). At both cell densities, the transcriptional response to SF/HGF was enhanced in the presence of ERK1, showing that activation of ERK1 by SF/HGF potentiated

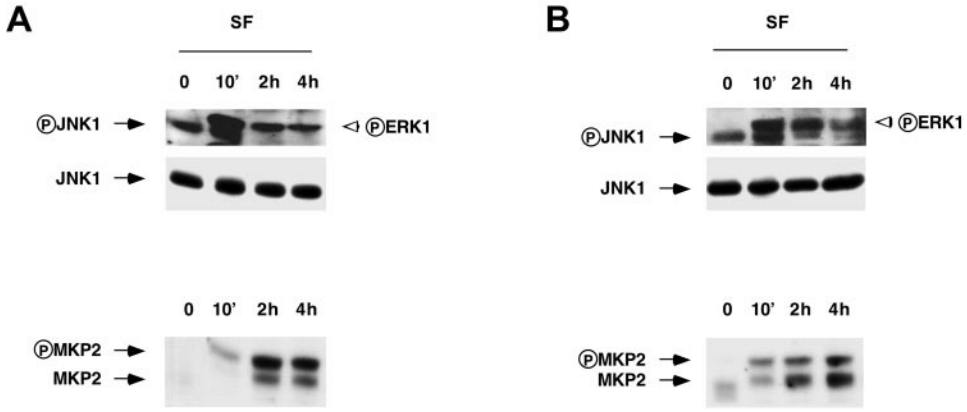

Figure 8. Effects of SF/HGF on phosphorylation of ERK and JNK and on expression of MKP2 at different cell densities. Cells were seeded on 100-mm plates (100,000 cells [A] and 400,000 cells [B]). The next day, the cells were incubated in DMEM–0.5% FCS. The following day, cells were treated for the indicated times with SF/HGF (30 ng/ml). Cell extracts and immunoblot analysis of phosphorylated JNK1 or MKP2 expression were performed as described in Figure 5. The filters were stripped and reprobed with the anti-JNK antibody.

this transcriptional response. In agreement with the results obtained with SF/HGF alone, SF/HGF was more efficient at inducing a transcriptional response in the presence of ERK1 at the lowest cell density (Figure 7, C and D). In contrast, JNK1 did not modify transcriptional activation by SF/HGF at the lowest cell density (Figure 7C), whereas it inhibited by 40% this transcriptional activation at the higher cell density (Figure 7D). In no case was JNK1 found to enhance the effect of SF/HGF.

We then investigated the effects of various dominant negative mutants, kinase-defective ERK1 ($ERK1^{TA}$) or JNK1 ($JNK1^{APF}$) and inactivated forms of RAS (RAS^{S186}) or CDC42 ($CDC42^{N17}$), which are upstream activators of ERK and JNK, respectively (Bagrodia *et al.*, 1995; Dhanasekaran and PremKumar Reddy, 1998). Inactive RAS and kinase-defective ERK1 were more efficient at inhibiting SF/HGF action at low density (Figure 7E) than at high density (Figure 7F). In contrast, at low density, inactive CDC42 and kinase-defective JNK1 had no effect (Figure 7E), whereas at high density they enhanced the response to SF/HGF (Figure 7F). The amplitude of the enhancing effects of inactive forms of CDC42 or JNK1 was variable, but these forms were never found to inhibit SF/HGF action. Thus, the inactive forms of these kinases or of their upstream activators gave results consistent with those obtained from their wild-type forms (Figure 7, compare C and D with E and F): low density favored the EBS/AP1-dependent transcriptional response to SF/HGF through the RAS-ERK pathway, and an inhibitory effect of JNK1 was seen only at high cell density.

We then transfected MKP1 and MKP2 and found distinct effects of these phosphatases on this transcriptional response. In particular, MKP2 potentiated both basal and SF/HGF-induced transcriptional responses at high cell density (Figure 7H). Hence, MKP2 behaves as $JNK1^{APF}$, the dominant negative inhibitor of JNK (Figure 7, E and F). In contrast, MKP1 abrogated the transcriptional response at both cell densities (Figure 7, G and H); it behaves like $ERK1^{TA}$, the dominant negative inhibitor of ERK (Figure 7, E and F).

Based on the demonstration that with time the phosphorylation of ERK, MKP2, and JNK are regulated differently by SF/HGF (Figures 2 and 4), we wished to test the hypothesis that at low cell density SF/HGF was more efficient at regulating their level of phosphorylation. Indeed, at the lowest cell density (Figure 8A), SF/HGF induced stronger phosphorylation of ERK at all times tested, as well as a stronger delayed dephosphorylation of JNK, than at high cell density (Figure 8B). For example, at low cell density, we did not detect any JNK phosphorylation at 2 and 4 h in the presence of SF/HGF (Figure 8A), whereas JNK phosphorylation was still detectable at the highest cell density (Figure 8B). Similarly, the phosphorylation of MKP2 was more pronounced at low density than at high cell density (Figure 8). These marked variations of phosphorylation of ERK, MKP2, and JNK indicate that the amplitude of activation of the RAS-MEK pathway is higher at a low rather than at a high cell density.

Together, these results show that at low cell density, efficient activation of the RAS-MEK pathway leads to strong induction of ERK and MKP2 phosphorylation and dephosphorylation of JNK and to efficient transcriptional response and cell scattering. In contrast, at high cell density, when the amplitude of activation of the RAS-MEK pathway is lower, a transrepressing effect of JNK is revealed and a complete scattering of the cells is not observed.

DISCUSSION

The signal transduction pathways implicating MAPKs convert signals received at the plasma membrane into the activation of intracellular targets, including transcription factors. In mammalian cells, the MAPKs ERK and JNK have been well investigated. In most cases, the emerging picture is that they belong to separate cascades that are activated independently and can target distinct transcription factors (Treisman, 1996; Widmann *et al.*, 1999). In this study, we found that through the same pathway, i.e., the RAS-RAF-MEK pathway, SF/HGF can regulate ERK phosphorylation, MKP2 phosphorylation, and JNK dephosphorylation as well as EBS/AP1-dependent transcriptional responses and cell

scattering. The functional consequence of this sequence of events was shown by measuring these responses at different cell densities. We found that at low density, a SF/HGF-RAS-MEK-ERK-MKP2 pathway is well activated and prevents a transrepressing effect of JNK, whereas at high cell density, this SF/HGF-RAS-MEK-ERK-MKP2 pathway is less efficiently activated and a transrepressing effect of JNK is observed.

SF/HGF Weakly Stimulates and Then Represses JNK Phosphorylation

We found that within a few minutes of stimulation, SF/HGF is a potent activator of ERK and a weak activator of JNK. These results are in agreement with those obtained with other growth factors acting through tyrosine kinase receptors (Minden *et al.*, 1994). In particular, similar characteristics of activation of ERK and JNK by SF/HGF were found in rat primary cultures of hepatocytes (Auer *et al.*, 1998) and in a human keratinocyte cell line (McCawley *et al.*, 1999), demonstrating that these results are not cell type specific. In contrast, TNF-α, which does not act through a tyrosine kinase receptor, is a weak activator of ERK and a potent activator of JNK in MDCK cells, as demonstrated in other cell types (Minden *et al.*, 1994; Auer *et al.*, 1998). This weak activation of JNK by SF/HGF was followed by a sustained repression for several hours, whereas ERK phosphorylation was still induced. This effect might not be restricted to SF/HGF, because VEGF was similarly shown to activate ERK and to inhibit JNK in human microvascular endothelial cells (Gupta *et al.*, 1999).

Previously, it was found that growth factors, including SF/HGF, can induce short-term activation of both ERK and JNK through distinct signaling pathways (Minden *et al.*, 1994; Garcia-Guzman *et al.*, 1999). For example, in PC12 cells, EGF or NGF activated two RAS-dependent MAPK cascades, one initiated by the RAF MAPK kinase kinase leading to ERK activation and the other initiated by the MEKK MAPK kinase kinase leading to JNK activation (Minden *et al.*, 1994). Our results with a pharmacological inhibitor of MEK did not contradict these findings and led us to further demonstrate that the same RAS-RAF-MEK pathway can cause both the rapid activation of ERK and the delayed repression of JNK. These results indicate that the mechanisms of short-term phosphorylation versus long-term dephosphorylation of JNK by the same growth factor can be distinct.

A likely mechanism for JNK dephosphorylation by SF/HGF involves the activation of dual-specificity phosphatases of the MKP family. Indeed, MKP family members are the products of immediate early genes, and several members of this family are transiently synthesized after activation of MAPKs (Camps *et al.*, 2000; Keyse, 2000). Because these MKP phosphatases have dual catalytic activity toward phosphotyrosine and phosphothreonine residues, they can in turn regulate the activity of MAPKs (Chu *et al.*, 1996; Brondello *et al.*, 1997; Hirsch and Stork, 1997; Keyse, 2000). In particular, MKP1 and MKP2, which share 60% amino acid sequence identity (Misra-Press *et al.*, 1995), can both be transiently induced by ERK or JNK and subsequently regulate their activity (Chu *et al.*, 1996; Brondello *et al.*, 1997; Hirsch and Stork, 1997). In our present study, MKP2, but not MKP1, was well detected and therefore more accessible for experimentation.

By examining MKP2 expression, we found that induction by SF/HGF of MKP2 phosphorylation (10 min) temporally precedes JNK dephosphorylation (1–4 h), whereas ERK dephosphorylation (4–8 h) occurs later. By using U0126, an inhibitor of the MEK kinase upstream of ERK, we further demonstrate a functional link between the rapid phosphorylation of both ERK and MKP2 and the delayed dephosphorylation of JNK. The fact that the MKP2 protein was barely detectable 4 h after treatment with U0126 suggests that this inhibitor either blocked its transcriptional induction or favored its degradation. Indeed, both mechanisms have been shown for regulation of MKP1 expression. In particular, activated ERK can reduce the degradation of MKP1, which is a labile protein targeted for degradation by the ubiquitin-directed proteasome complex (Brondello *et al.*, 1999). The mechanisms regulating MKP2 expression downstream of activated ERK in MDCK cells await further clarification.

Activation of JNK Can Inhibit Specific Transcriptional Responses to SF/HGF

As reported in the INTRODUCTION, the ERK and JNK MAPKs are involved in various biological responses to SF/HGF (Rodrigues *et al.*, 1997; Auer *et al.*, 1998; Garcia-Guzman *et al.*, 1999; McCawley *et al.*, 1999; Ried *et al.*, 1999). For example, ERK is involved in epithelial cell scattering (Potempa and Ridley, 1998; Tanimura *et al.*, 1998; Tulasne *et al.*, 1999), whereas JNK is involved in proliferation of hepatocytes (Auer *et al.*, 1998) or in transformation of FR3T3 fibroblast cells transfected with the oncogenic TPR-MET receptor (Rodrigues *et al.*, 1997). This raises the question of whether these two kinases can be involved in the same biological responses induced by SF/HGF. We tested this hypothesis by investigating their effects on the same transcriptional response implicating an EBS/AP1 response element, which was initially identified as a functional RAS-responsive element in the polyomavirus enhancer (Wasylyk *et al.*, 1990). Since then, a number of similar response elements have been identified in regulatory regions of various cellular genes, including protease genes (Wasylyk *et al.*, 1998).

We found that the RAS-ERK pathway induces transcriptional activation through this EBS/AP1 response element, but that JNK1 does not. Rather, JNK1 had no effect or an inhibitory effect, depending on cell density. This extends our previous work, in which we showed that SF/HGF and RAS activated this transcriptional response (Fafeur *et al.*, 1997) and that a dominant negative mutant of CDC42 does not impair, but rather favors, transactivation induced by chimeric MET receptors (Tulasne *et al.*, 1999). It was reported previously in NIH-3T3 fibroblast cells transfected with a MET receptor that the uPA promoter can be stimulated by a signal transduction pathway involving RAS-RAF-MEK-ERK but not JNK (Ried *et al.*, 1999). Whereas these authors concluded that JNK is not involved in mediating uPA promoter induction, our data demonstrate that JNK1 modulates the efficiency of the RAS-RAF-MEK-ERK signal transduction pathway.

It can be argued that our transactivation results, including transrepression by JNK, were observed after enforced expression of ERK or JNK or of their possible upstream activators. To examine the effects of ERK and JNK in more physiological conditions, we also performed transactivation

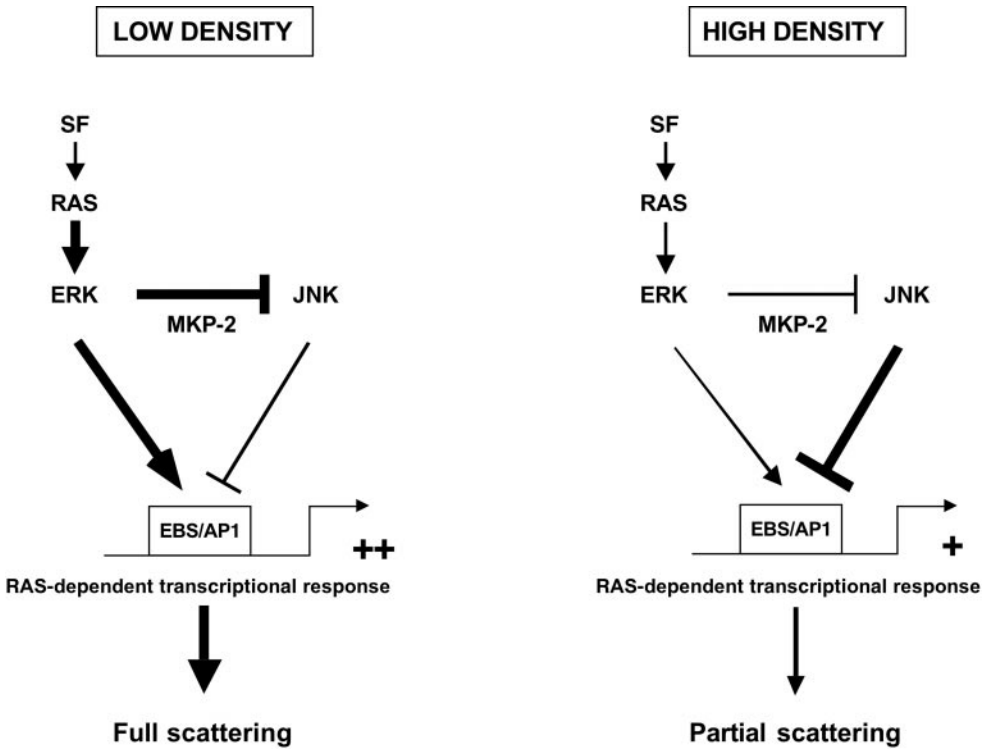

Figure 9. Model of SF/HGF signaling implicating ERK, MKP2, and JNK in MDCK cells at low and high cell density. See text for details.

assays in the presence of TNF-α, which activates JNK strongly and ERK weakly in many cells, including MDCK epithelial cells. We found that TNF-α inhibited the ability of SF/HGF to induce this transcriptional response (our unpublished results), demonstrating that a potent endogenous activator of JNK can also inhibit transcriptional responses to SF/HGF. It is clear that the finding of a negative role for JNK in transmitting signal transduction SF/HGF is original, because most studies emphasize the possible positive involvement of JNK in signal transduction. Nonetheless, it was similarly demonstrated that insulin action involves a delayed and sustained inhibition of JNK (Desbois-Mouthon *et al.*, 2000). The main difference between that work and our study is that those authors proposed a mechanism for JNK inhibition implicating the phosphatidylinositol 3-kinase, whereas we explored a mechanism that involves activation of the RAS-MEK-ERK pathway.

A SF/HGF-RAS-RAF-MEK-ERK-MKP2 Pathway Can Prevent Transrepression by JNK

Finally, we show that the activation of ERK and MKP2 and the subsequent inactivation of JNK by SF/HGF are functionally involved in mediating transcriptional activation of an EBS/AP1 promoter element that correlates with efficient scattering in MDCK cells. This was shown by measuring these responses at different cell densities, because it was our common observation that low cell density favors both the EBS/AP1 transactivation and scattering in response to SF/HGF. At low cell density, SF/HGF efficiently induces this 1transcriptional response through RAS, ERK, and MKP2, whereas JNK has no effect. The strong activation of RAS, ERK, and MKP2 leads to the dephosphorylation of JNK and explains the absence of transrepression by JNK. At higher cell density, SF/HGF is less efficient at inducing this transcriptional response through RAS, ERK, and MKP2, and JNK has a transrepressing effect. The weak activation of RAS, ERK, and MKP2 leads to a less efficient dephosphorylation of JNK and reveals the transrepressing effect of JNK. The role of JNK in this cascade of events, therefore, is to modulate the efficiency of a RAS-RAF-MEK-ERK signal transduction pathway. The corresponding model of SF/HGF signaling implicating ERK, MKP2, and JNK at different cell densities is depicted in Figure 9.

Various studies have demonstrated that quantitative differences are found between proliferative and differentiation signals (Marshall, 1995). In particular, it is clear that sustained versus transient activation of ERK represents an underlying mechanism to account for tyrosine kinase receptor specificity in ligand-induced specific biological responses. For example, in PC12 cells, NGF promotes sustained activation of ERK for several hours and differentiation, i.e., neurite outgrowth, whereas EGF promotes transient activation of ERK and proliferation (Traverse *et al.*, 1992). Similarly, in a keratinocyte cell line, EGF and SF/HGF induces sustained activation of ERK and stimulates MMP-9 induction and migration, whereas IGF and KGF transiently activate ERK and are mitogenic (McCawley *et al.*, 1999). A similar mechanism implicating activated JNK has not been shown, perhaps because these growth factors are weak activators of

JNK. Alternatively, our results indicate that both sustained activation of ERK and repression of JNK may account for the scattering signal induced by SF/HGF.

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REFERENCES

Auer, K.L., *et al.* (1998) The Ras/Rac1/Cdc42/SEK/JNK/c-Jun cascade is a key pathway by which agonists stimulate DNA synthesis in primary cultures of rat hepatocytes. Mol. Biol. Cell *9*, 561–573.

Bagrodia, S., Dérijard, B., Davis, R.J., and Cerione, R.A. (1995). Cdc42 and PAK-mediated signaling leads to jun kinase and p38 mitogen-activated protein kinase activation. J. Biol. Chem. *270*, 27995–27998.

Beauchemin, N., Kunath, T., Robitaille, J., Chow, B., Turbide, C., Daniels, E., and Veillette, A. (1997). Association of biliary glycoprotein with protein tyrosine phosphatase SHP-1 in malignant colon epithelial cells. Oncogene *14*, 783–790.

Brondello, J.M., Brunet, A., Pouyssegur, J., and McKenzie, F.R. (1997). The dual specificity mitogen-activated protein kinase phosphatase-1 and -2 are induced by the p42/p44MAPK cascade. J. Biol. Chem. *272*, 1368–1376.

Brondello, J.M., Pouyssegur, J., and McKenzie, F.R. (1999). Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. Science *286*, 2514–2517.

Camps, M., Nichols, A., and Arkinstall, S. (2000). Dual specificity phosphatases: a gene family for control of MAP kinase function. FASEB J. *14*, 6–16.

Cano, E., and Mahadevan, L.C. (1995). Parallel signal processing among mammalian MAPKs. Trends Biochem. Sci. *20*, 117–122.

Chu, Y., Solski, P.A., Khosravi-Far, R., Der, C.J., and Kelly, K. (1996). The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP- 2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation. J. Biol. Chem. *271*, 6497–6501.

Coso, A., Chiarello, M., Yu, J.C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, S. (1995). The small GTP-binding proteins RAC1 and CDC42 regulate the activity of the JNK/SAPK signaling pathway. Cell *81*, 1137–1146.

Derijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R.J. (1994) JNK: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell *76*, 1025–1037.

Desbois-Mouthon, C., Cadoret, A., Blivet-Van Eggelpoel, M.J., Bertrand, F., Caron, M., Atfi, A., Cherqui, G., and Capeau, J. (2000). Insulin-mediated cell proliferation and survival involve inhibition of c-Jun N-terminal kinases through a phosphatidylinositol 3-kinase- and mitogen-activated protein kinase phosphatase-1-dependent pathway. Endocrinology *141*, 922–931.

Dhanasekaran, N., and PremKumar Reddy, E. (1998). Signaling by dual specificity kinases. Oncogene *17*, 1447–1455.

Fafeur, V., Tulasne, D., Queva, C., Vercamer, C., Dimster, V., Mattot, V., Stehelin, D., Desbiens, X., and Vandenbunder, B. (1997). The ETS1 transcription factor is expressed during epithelial-mesenchymal transitions in the chick embryo and is activated in scatter factor-stimulated MDCK epithelial cells. Cell Growth Differ. *8*, 655–665.

Fanger, G.R., Lassignal Johnson, N., and Johnson, G.L. (1997). MEK kinases are regulated by EGF and selectively interact with Rac/Cdc42. EMBO J. *16*, 4961–4972.

Favata, M.F., *et al.* (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. *273*, 18623–18632.

Garcia-Guzman, M., Dolfi, F., Zeh, K., and Vuori, K. (1999). Met-induced JNK activation is mediated by the adapter protein crk and correlates with the gab1-crk signaling complex formation. Oncogene *18*, 7775–7786.

Guo, Y.L., Baysal, K., Kang, B., Yang, L.J., and Williamson, J.R. (1998). Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. J. Biol. Chem. *273*, 4027–4034.

Gupta, K., Kshirsagar, S., Li, W., Gui, L., Ramakrishnan, S., Gupta, P., Law, P.Y., and Hebbel, R.P. (1999). VEGF prevents apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ERK and SAPK/JNK signaling. Exp. Cell Res. *247*, 495–504.

Hartmann, G., Weidner, K.M., Schwarz, H., and Birchmeier, W. (1994). The motility signal of scatter factor/hepatocyte growth factor mediated through the receptor tyrosine kinase Met requires intracellular action of ras. J. Biol. Chem. *269*, 21936–21939.

Hirsch, D.D., and Stork, P.J. (1997). Mitogen-activated protein kinase phosphatases inactivate stress-activated protein kinase pathways in vivo. J. Biol. Chem. *272*, 4568–4575.

Keyse, S.M. (2000). Protein phosphatases and the regulation of mitogen-activated protein kinase signaling. Curr. Opin. Cell Biol. *12*, 186–192.

Keyse, S.M., and Emslie, E.A. (1992). Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. Nature *359*, 644–647.

King, A.G., Ozanne, B.W., Smythe, C., and Ashworth, A. (1995). Isolation and characterization of a uniquely regulated threonine, tyrosine phosphatase (TYP 1), which inactivates ERK2 and p54jnk. Oncogene *11*, 2553–2563.

Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell *80*, 179–185.

McCawley, L.J., Li, S., Wattenberg, E.V., and Hudson, L.G. (1999). Sustained activation of the mitogen-activated protein kinase pathway: a mechanism underlying receptor tyrosine kinase specificity for matrix metalloproteinase-9 induction and cell migration. J. Biol. Chem. *274*, 4347–4353.

Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Dérijard, B., Davis, R.J., Johnson, G.L., and Karin, M. (1994). Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science *266*, 1719–1723.

Misra-Press, A., Rim, C.S., Yao, H., Roberson, M.S., and Stork, P.J. (1995). A novel mitogen-activated protein kinase phosphatase: structure, expression, and regulation. J. Biol. Chem. *270*, 14587–14596.

Nguyen, L., Holgado, M.M., Maroun, C., Fixman, E.D., Kamikura, D., Fournier, T., Charest, A., Tremblay, M.L., Wong, A.J., and Park, M. (1997). Association of the multisubstrate docking protein Gab1 with the hepatocyte growth factor receptor requires a functional

Grb2 binding site involving tyrosine 1356. J. Biol. Chem. *272*, 20811–20819.

Pelicci, G., *et al.* (1995). The motogenic and mitogenic responses to HGF are amplified by the SHC adaptor protein. Oncogene *10*, 1631–1638.

Pepper, M.S., Matsumoto, K., Nakamura, T., Orci, L., and Montesano, R. (1992). Hepatocyte growth factor increases urokinase-type plasminogen activator (u-PA) and u-PA receptor expression in Madin-Darby canine kidney epithelial cells. J. Biol. Chem. *267*, 20493–20496.

Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graaziani, A., Panayotou, G., and Comoglio, P.M. (1994). A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell *77*, 261–271.

Potempa, S., and Ridley, A.J. (1998). Activation of both MAP kinase and phosphatidylinositide 3-kinase by Ras is required for hepatocyte growth factor/scatter factor-induced adherens junction disassembly. Mol. Biol. Cell *9*, 2185–2200.

Ried, S., Jager, C., Jeffers, M., Vande Woude, G.F., Graeff, H., Schmitt, M., and Lengyel, E. (1999). Activation mechanisms of the urokinase-type plasminogen activator promoter by hepatocyte growth factor/scatter factor. J. Biol. Chem. *274*, 16377–16386.

Rodrigues, G.A., Park, M., and Schlessinger, J. (1997). Activation of the JNK pathway is essential for transformation by the Met oncogene. EMBO J. *16*, 2634–2645.

Rorth, P., Nerlov, C., Blasi, F., and Johnsen, M. (1990). Transcription factor PEA3 participates in the induction of urokinase plasminogen activator transcription in murine keratinocytes stimulated with epidermal growth factor or phorbol-ester. Nucleic Acids Res. *18*, 5009–5017.

Su, B., and Karin, M. (1996). Mitogen-activated. protein kinase cascades and regulation of gene expression. Curr. Opin. Immunol. *8*, 402–411.

Tanimura, S., Chatani, Y., Hoshino, R., Sato, M., Watanabe, S.-I., Kataoka, T., Nakamura, T., and Kohno, M. (1998). Activation of the 41/43 kDa mitogen-activated protein kinase signaling pathway is required for hepatocyte growth factor-induced cell scattering. Oncogene *17*, 57–65.

Traverse, S., Gomez, N., Paterson, H., Marshall, C., and Cohen, P. (1992). Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells: comparison of the effects of nerve growth factor and epidermal growth factor. Biochem. J. *288*, 351–355.

Treisman, R. (1996). Regulation of transcription by MAP kinase cascades. Curr. Opin. Genet. Dev. *4*, 96–101.

Tulasne, D., Paumelle, R., Weidner, K.M., Vandenbunder, B., and Fafeur, V. (1999). The multisubstrate docking site of the MET receptor is dispensable for MET-mediated RAS signaling and cell scattering. Mol. Biol. Cell *10*, 551–565.

Wasylyk, B., Hagman, J., and Gutierrez-Hartmann, A. (1998). Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem. Sci. *23*, 213–216.

Wasylyk, B., Wasylyk, C., Flores, P., Begue, A., Leprince, D., and Stéhelin, D. (1990). The c-ets proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. Nature *346*, 191–193.

Weidner, K.M., Dicesare, S., Sachs, M., Brinkmann, V., Behrens, J., and Birchmeier, W. (1996). Interaction between gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. Nature *384*, 173–176.

Weidner, K.M., Sachs, M., and Birchmeier, W. (1993). The Met receptor tyrosine kinase transduces mobility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. J. Cell Biol. *121*, 145–154.

Widmann, C., Gibson, S., Jarpe, M., and Johnson, G. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. *79*, 143–180.