Hepatocyte growth factor signalling stimulates hypoxia inducible factor-1 (HIF-1) activity in HepG2 hepatoma cells

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Hepatocyte growth factor (HGF), a multifunctional cytokine of mesenchymal origin, activates the DNA binding of hypoxia inducible factor-1 (HIF-1) in the HepG2 cell line: the activated complex contained the inducible α subunit. An increased expression of HIF-1α (mRNA and nuclear protein levels) was observed. To investigate the molecular basis of the HIF-1 response under this non-hypoxic condition, we evaluated first the expression of putative target genes. We found a time-dependent increase in steady-state mRNA levels of heme oxygenase and urokinase plasminogen activator at 4 h, followed by that of urokinase receptor at 10 h. The enhanced expression of these genes might confer the invasive phenotype, since HGF is a proliferative and scatter factor. Second, we examined some aspects of HIF-1 activity regulation in HGF-treated cells with the following findings: (i) the activation of HIF-1 DNA binding was prevented by proteasome blockade, probably because stabilization of the cytosolic α-subunit protein level is not sufficient to generate a functional form: also under these conditions nuclear protein level of HIF-1α did not increase; (ii) *N*-acetylcysteine, a free radical scavenger, strongly decreased HIF-1 activation suggesting a role of reactive oxygen species in this process; (iii) the thiol reducing agent dithiothreitol was ineffective. Third, consistent with these data, *N*-acetylcysteine reduced the stimulatory effect of HGF on stress kinase activities, while p42/44 mitogen activated kinase (MAPK) was unmodified, suggesting an involvement of c-Jun-N-terminal kinase (JNK) and p38 MAPK in HIF-1 activation. Finally, LY 294002 induced the blockade of phosphatidylinositol 3-kinase (PI3K), one of the principal transducers of HGF/Met receptor signalling, prevented the enhancement of HIF-1 DNA binding and JNK activity, but the inhibition of p42/44 MAPK phosphorylation with PD 98059 was ineffective. In conclusion, we suggest that HGF triggers a signal transduction cascade involving PI3K and ultimately activates HIF-1.

Introduction

Hepatocyte growth factor (HGF) is a mesenchymal cell-derived cytokine which induces a spectrum of biological activities,

Abbreviations: GSH, reduced glutathione; HGF, hepatocyte growth factor; HIF-1, hypoxia-inducible factor; Hox, heme oxygenase; HRE, hypoxia responsive element; JNK/SAPK, c-Jun-N-terminal kinase/stress activated protein kinase; MAPK, mitogen activated protein kinase; Oct-1, octamer-1; PI3K, phosphatidylinositol 3-kinase; rhHGF, recombinant human HGF; ROS, reactive oxygen species; uPA, urokinase plasminogen activator; uPAR, urokinase receptor.

including modulation of mitogenesis, stimulation of motility and promotion of matrix invasion *in vitro* (1–5) and angiogenesis *in vivo* (6). Met transmembrane tyrosine kinase is the high affinity receptor for HGF. The C-terminal domain includes two critical tyrosine residues that when phosphorylated form a specific docking site for multiple signal transducers and adaptors. Signalling through HGF/Met promotes a cellular program for invasiveness *in vitro* that integrates apparently independent biological responses: phosphatidylinositol 3-kinase (PI3K) mediates cell dissociation and migration ('scattering' activity) and the Grb2/SoS/Ras pathway controls the proliferative effect (7,8). It has been reported that in some types of cancer cell line of epithelial and mesenchymal origin, and in NIH 3T3 cells stably transfected with Met receptor to obtain an autocrine loop, HGF stimulates the secretion of proteases which destroy the basement membrane *in vitro*, such as matrix metalloproteinases and serine protease urokinase (9–12). Small G-proteins of the Rho family downstream of Ras seem to be involved in the regulation of the urokinase-type plasminogen activator (uPA) promoter through AP-1 activity (12). Inappropriate HGF/Met signalling leads to the development of tumours and metastases. Met is mutated in a small number of genetically inherited tumours (13), but is overexpressed at high frequency in sporadic cancer (14), and is amplified during the progression from primary tumour to metastatic cells (3,15). Recently, a more direct link between paracrine effects of HGF and motility/invasion at the secondary site of tumour development has been reported (16).

We have previously observed that the intracellular signal transduction cascade(s) triggered by HGF/Met binding in human hepatoma cells HepG2 activate(s) downstream transcription factors involved in growth and apoptosis, such as AP-1 and c-Myc/Max (17). Consistent with these data, in the same experimental model HGF causes an initial stimulation of DNA synthesis before the occurrence of a cytostatic and apoptotic effect (18,19). Taking also into account the rather surprising activation of NF-κB, known to regulate transcription of genes under stress, injury and inflammatory conditions (17), further studies were then undertaken to clarify specific transduction pathways ultimately leading to transcription factor activation possibly implicated in the invasive phenotype induced by HGF.

The present study deals with the HGF-mediated activation of hypoxia-inducible factor-1 (HIF-1), a transcription factor which plays a central role in tumour progression through metabolic adaptation and angiogenesis (20). HIF-1 is a heterodimeric protein consisting of inducible α and constitutive β subunits, that contain N-terminal basic–helix–loop–helix–PAS domains required for dimerization and DNA binding (21). HIF-1α protein level is elevated in the majority of common human cancers and their metastases, due to the presence of intratumoural hypoxia and as a result of mutations in genes encoding oncoproteins and tumour suppressors (21). The HIF-1α protein is also highly expressed in cells treated with insulin-

like growth factor 1 and 2, fibroblast growth factor 2 or epidermal growth factor in relationship to cell proliferation (21) and HIF-1 DNA binding is stimulated by pro-inflammatory cytokines or insulin (22,23). Here, we also evaluated under HGF stimulation the expression of putative HIF-1 target genes as well as some of the possible molecular mechanisms involved in HIF-1 DNA binding. The genes examined were heem oxygenase (Hox), whose reaction product carbon monoxide modulates HIF-1 activity (21,24), urokinase receptor (uPAR) (25,26) and uPA (27), because surface plasminogen activation may give an advantage to malignant cells by conferring invasive and metastatic properties (26). The involvement of proteasome and redox state as well as the role of mitogen activated protein kinase (MAPK) and PI3K signal transducers in the regulation of HIF-1 activation and HIF-1α nuclear protein level after HGF treatment have been investigated. The rationale is that HIF-1α regulation seems to be generally at the post-translational level via degradation/stabilization and/or phosphorylation. The findings indicating the main role played by PI3K possibly through c-Jun-N-terminal kinase/stress activated protein kinase (JNK/SAPK) in HGF-induced HIF-1 activation are discussed.

Materials and methods

Materials

Minimal essential medium, fetal bovine serum and *N*-acetyl-L-cysteine were from Sigma (St Louis, MO). Recombinant human HGF (rhHGF) was from R&D System Europe (Abingdon, UK). [γ-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), Hybond C-extra nylon filters and Hybond ECL nitrocellulose membranes were from Amersham (Little Chalfont, Bucks, UK). Monoclonal anti-human HIF-1α antibody (OZ15) was from NeoMarkers (Union City, CA). Monoclonal anti-HIF-1α antibody H1α67, which was protein-G purified from cell max culture supernatant, was purchased from Novus Biological (Littelton, CO). Goat polyclonal antibody against c-jun (N) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HGF chicken anti-mouse polyclonal antibody (titre 1:40 000) was a generous gift of Dr Rusciano (Friedrich Miescher Institute, Basel) (17). JNK/SAPK, p38 MAPK and p42/44 MAPK assay kits were purchased from New England Biolabs (Beverly, MA). *clasto*-Lactacystin β-lactone, PD 98059 and LY 294002 were from Calbiochem (San Diego, CA). All the other chemicals used were of the highest grade available.

Cell culture and treatments

HepG2 cells (European Collection of Cell Cultures) were cultured in minimal essential medium as reported (18). For the experiments, cells were serum starved (0.1% fetal bovine serum) for 24 h and then the medium was replaced with fresh medium without serum but containing 40 ng/ml rhHGF. The rhHGF preparation used was >95% heterodimeric active form. When the cells were treated with the inhibitors, they were added to the cultures before HGF, as reported in the Results section. In some experiments, the effect of 10% FBS or 40 ng/ml HGF in the presence or absence of 20 µg/ml anti-HGF antibody was examined. This anti-HGF antibody is mouse and human reactive (17). For the inhibitors dissolved in DMSO, the final concentration of DMSO was between 0.02 and 0.2%, a dose which is non-toxic (18). For hypoxic stimulation, subconfluent cells (growing in 10% fetal bovine serum-containing medium) were placed in an incubator chamber that was thoroughly flushed with a gas mixture containing 5% CO_2, 1% O_2 and nitrogen-balanced, tightly sealed and incubated at 37°C for 4 h (28).

Northern blot analysis

Total cellular RNA was extracted by a single step method (29) and equal amounts (30 µg) were electrophoresed on 0.8% agarose formaldehyde gels. To confirm that each lane contained equal amounts of total RNA, the ribosomal RNA concentration in each lane was estimated visually by ethidium bromide staining. RNA was transferred to nylon filters by capillary blotting and was hybridized with the following specific probes labelled by nick translation (Amersham): a plasmid containing human HIF-1α cDNA (kindly provided by R.D.Thornton), a 1.5 kb *Pst*I fragment from uPA human cDNA and a 1.4 kb *Xho*I fragment from uPAR human cDNA (kindly provided by F.Blasi, DIBIT, Milan, Italy), and a plasmid containing the human cDNA for Hox (kindly provided by R.M.Tyrrell, University of Bath, UK).

Electrophoretic mobility shift assay (EMSA)

The EMSA method was used to analyse the DNA-binding activities of HIF-1 and Octamer-1 (Oct-1) transcription factors in nuclear extracts (30), prepared from 30×10^6 cells (pool of two flasks) in the presence of the tyrosine phosphatase inhibitor orthovanadate (22,28,30,31). Protein concentration was determined by Bio-Rad assay with BSA as standard. Single-stranded oligo-nucleotides with consensus binding-sites of trancription factors were labelled with T4 polynucleotide kinase (Amersham) by using [γ-^{32}P]ATP, were annealed to the complementary strand and were purified by polyacrylamide gel electrophoresis. Nuclear proteins (20 µg) were incubated for 20 min at 25°C in the binding reaction mixtures, containing 0.5 ng ^{32}P-labelled double-stranded sequence. For competition experiments, 50-fold excess of the specific or unspecific unlabelled double-stranded sequence was added to the binding mixtures. DNA–protein complexes were resolved by electrophoresis on 5% polyacrylamide gel at 4°C in running buffer (30) and the gels were dried and autoradiographed. For supershift assays, 20 µg nuclear extracts were incubated with 1 µg anti-HIF-1α (OZ15) or anti-c-jun antibody for 60 min on ice without the oligonucleotide, followed by incubation with the labelled oligo-nucleotide and then electrophoresed as described above.

The sequence of the oligonucleotide containing the Hypoxia responsive element (HRE), present in the promoter of erythropoietin (HIF-1 target gene), was 5′-GCCCTACGTGCTGTCTCA (30); Oct-1 binding site was 5′-TGCGAATGCAAATCACTAGAA-3′ (32). These oligonucleotides were synthesized by Primm (Milan, Italy).

Immunoblot assay

After preparation of nuclear (30,33) and cytosolic (34) extracts, aliquots corresponding to 20 and 50 µg protein, respectively, were fractionated by 7% SDS–PAGE and transferred to a nitrocellulose membrane (19). Blots were incubated with a 1:1000 dilution of anti-HIF-1α monoclonal antibody H1α67, followed by a sheep antimouse immunoglobulin (1:3000) and detected by ECL plus reagent (Amersham).

Determination of JNK/SAPK, p38 MAPK and p42/44 MAPK activities

The protein kinase activities were determined in lysates prepared from 10×10^6 cells by using New England Biolabs kits, according to the manufacturer's instructions. JNKs were separated from cell lysates (corresponding to 500 µg protein) using GST–c-Jun (1–89) and the kinase reaction was performed in the presence of cold ATP. Phospho-P38 MAPK and phospho-p42/44 MAPK were immunoprecipitated from cell lysates (corresponding to 200 µg protein) with specific immobilized antibodies, and *in vitro* activity assays were carried out with cold ATP in the presence of the respective substrates, i.e. ATF-2 fusion protein (19–96) and Elk-1 fusion protein (307–428). Phosphorylation of the substrates was evaluated by western blot (in 10% SDS–PAGE) and hybridization was performed by using anti-phospho-c-Jun (Ser63), anti-phospho-ATF-2 (Thr71) or anti-phospho-Elk-1 (Ser383) antibodies. The immunoblots were developed with ECL kit and were exposed to X-ray films.

Densitometric analysis

All the autoradiograms were quantified by densitometric analysis (OD/mm^2) using a LKB Image Master DTS system (Amersham Pharmacia Biotech).

Statistical analysis

Densitometric values were analysed by analysis of variance, with $P < 0.05$ considered significant.

Results

HGF activates HIF-1 DNA binding by increasing HIF-1α expression

Hepatocyte growth factor triggers a unique biological program leading to invasive growth. First, we examined in HepG2 hepatoma cells, that undergo scatter effect and proliferate in response to HGF (18,35), the DNA binding activity of HIF-1 and the expression of HIF-1α protein and mRNA (Figure 1). The formation of HRE-binding complex(es) was evaluated in HepG2 cells exposed either to HGF after starvation or to hypoxia (Figure 1A). Nuclear extracts were allowed to react with the specific radiolabelled oligonucleotide, corresponding to the HRE of the erythropoietin gene (30) and the mixtures were subjected to EMSA. At 2 h, HGF induced the formation of a HIF-1 complex, that increased progressively up to 6 h and disappeared almost completely at 14 h. In addition, constitutive bands with faster mobilities appeared in EMSA
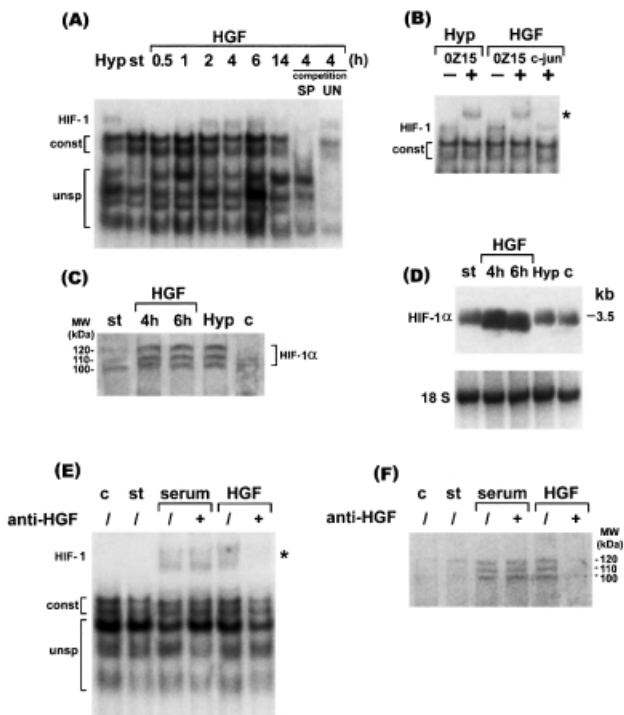
Fig. 1. Time-course of HIF-1 DNA binding and HIF-1α expression in HGF-treated cells. HepG2 cells were serum-starved (st) and then treated with 40 ng/ml HGF or 10% FBS for the indicated times. In some experiments, cultures of subconfluent cells, growing in 10% FBS (c), were exposed to hypoxic (Hyp) conditions (1% O$_2$ for 4 h). (**A**) Electrophoretic mobility shift assay of nuclear protein binding to the HRE sequence. Nuclear extracts (corresponding to 20 μg protein) were incubated with the labelled oligonucleotide probe. Inducible (HIF-1), constitutive (const) and unspecific (unsp) protein–DNA complexes are indicated. The last two lanes show specific (SP) and unspecific (UN) competitions with unlabelled HRE and Oct-1 sequences, respectively. (**B**) Super gel-shift analysis of DNA binding complexes, using nuclear protein extracts from cells exposed to Hyp (4 h) or HGF (6 h) treatment and the specific anti-HIF-1α (OZ15) or unspecific anti-c-jun antibodies. An asterisk indicates the position of the supershift complex. (**C**) Western blot of nuclear extracts separated by SDS–PAGE (7% gel) and blotted with anti HIF-1α antibody (clone H1α67). (**D**) Northern blot of total RNA (30 μg) probed with radiolabelled cDNA corresponding to HIF-1α transcript. 18 S rRNA was used as a control for RNA loading and for normalization of the densitometric values. (**E**) Super gel-shift analysis of DNA binding complexes and (**F**) western blot analysis performed as reported above, using nuclear extracts from cells treated for 6 h with 10% FBS or HGF in the presence or absence of anti-HGF antibody. An asterisk indicates the position of the supershift complex. The autoradiograms shown are representative of at least three different experiments.

both in starved and in HGF-treated cells. The mobility of the inducible and the constitutive binding complexes after HGF treatment was the same as that observed after hypoxia. It is noteworthy that in starved cells, as already known for normoxic subconfluent cells (28), the specific HIF-1 complex could not be detected. The specificity of the HIF-1 complex was demonstrated by competition with unlabelled HRE or the unrelated oligonucleotide Oct-1. Excess amount of unlabelled HRE (specific competition) completely abolished the formation of inducible HIF-1 and constitutive complexes, whereas the unspecific bands were unaffected. Excess amount of unlabelled Oct-1 (unspecific competition) had no effect on the inducible and constitutive bands, while the unspecific bands disappeared. Super gel-shift experiments (Figure 1B) with the specific anti-HIF-1α antibody (OZ15) demonstrated that the complex induced by HGF contained this subunit, whose expression is

known to be tightly regulated by the cellular O$_2$ concentration (21). Unspecific anti-c-jun antibody did not cause supershift of the inducible band. DNA binding activity of the constitutively expressed ubiquitous Oct-1 transcription factor was unaffected by either starvation, HGF or hypoxia (data not shown).

In an attempt to improve our understanding of the regulation of HIF-1α under the experimental conditions reported above, protein and mRNA levels in HGF-treated cells were evaluated (Figure 1C and D). Nuclear extracts were prepared from starved cells treated with HGF or from subconfluent cells (10% fetal bovine serum) exposed to hypoxia and subjected to immunoblot assay using a monoclonal antibody specific for HIF-1α (clone H1α67) (32). As reported for various cell lines (34), the electrophoretic migration pattern of HIF-1α was very diffuse (104–116 kDa) (Figure 1C). It has been suggested that this is caused by the post-translational modification of HIF-1α. We observed that protein levels of the α subunit augmented 4 and 6 h after HGF as well as in 4 h hypoxia treated-cells, and normoxic control levels were similar to those of starved cells. Steady-state mRNA levels of HIF-1α were examined by northern blot (Figure 1D) and densitometric analysis of three separate experiments was performed. HIF-1α transcript level was up-regulated (3.2 ± 0.5-fold) 4 h after treatment of starved cells with HGF, declining at 6 h (2.1 ± 0.4-fold). For the band corresponding to HIF-1α mRNA level in starved cell, the mean of densitometric values of three separate experiments was 71.1 ± 8.7 (OD for area in mm^2). HIF-1α mRNA level of normoxic control was similar to that of starved cells, and was unchanged by hypoxia treatment. To demonstrate the specificity of HIF-1α induction after HGF, we performed EMSA and western blot analysis after 10% serum addition to starved cells in the presence or absence of anti-HGF antibody. In Figure 1E, we show that serum treatment (6 h) activated HIF-1 DNA binding, that was unmodified by anti-HGF antibody. This result was consistent with the pattern of nuclear HIF-1α protein level (Figure 1F) showing some degree of expression in starved cells that increased in response to 6 h-serum stimulation in the presence or the absence of anti-HGF antibody. It is worth noting that the dose of antibody used here abolished the stimulatory effect of HGF on HIF-1 DNA binding (Figure 1E) and α subunit protein level (Figure 1F).

HGF increases the expression of HIF-1 target genes

To examine the ultimate molecular targets of HGF/Met signalling, the mRNA expression of target genes containing HRE sequences, such as Hox and uPAR, were determined (25,36). Because uPA is recruited on the cell surface by its specific receptor, we asked also whether uPA mRNA expression was regulated by HGF. The analysis of the uPA gene 5′-flanking region (GenBank accession no. Y11873) shows five potential HRE sequences, each of which differs by only two nucleotides from the consensus sequence 5′-XACGTGCX-3′, originally identified in the gene encoding erythropoietin (30). Three sequences are 5′-XGCGTGAX-3′, and two sequences are 5′-XGCGTGGX-3′. Formation of plasmin localized to the cell membrane may be important for tumour invasiveness, and HIF-1 activity is probably related to biological processes in which migration and invasion occur (25,27).

The typical experiment reported in Figure 2 shows that HGF increased the steady-state mRNA levels of Hox and uPA at 4 h (about 3- and 8-fold relative to starved cells) that rapidly decreased thereafter. uPAR mRNA level tripled at 10 h and
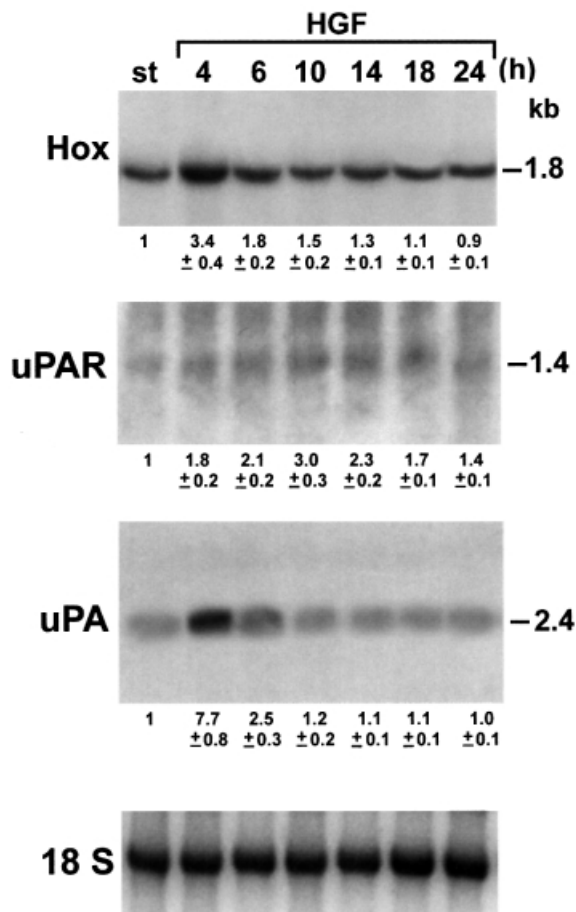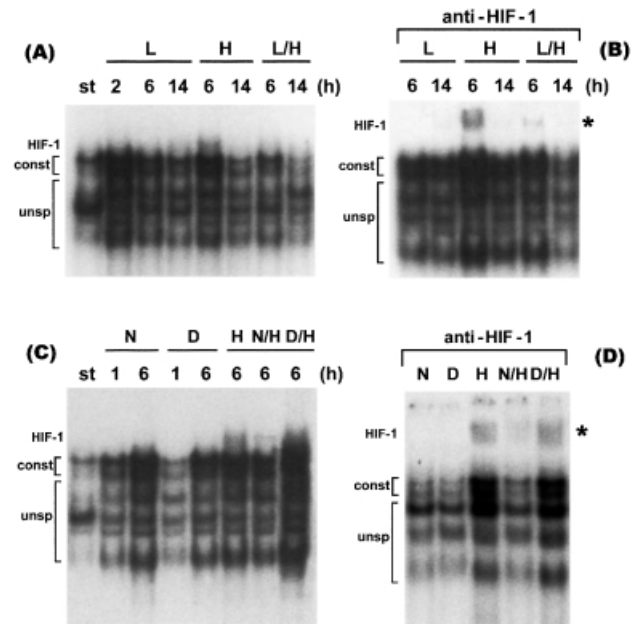
Fig. 2. mRNA expression of HIF-1 target genes. Serum starved HepG2 cells were stimulated for different time periods with 40 ng/ml HGF. Samples (30 µg) of total cellular RNA from these cells were analysed by northern blot and hybridized with ^{32}P-labelled probes for heme oxygenase (*Hox*), urokinase receptor (*uPAR*) and urokinase plasminogen activator (*uPA*). 18 S rRNA was used as a control for RNA loading and for normalization of the densitometric values. The values at the bottom of the respective northern blots indicate the fold increases ± SE of steady-state mRNA levels in HGF-treated cells relative to that in starved cells (st). The results shown are representative of three independent experiments.

Fig. 3. Proteasome blockade and reducing agents influence HGF-induced HIF-1 DNA binding. (**A**) Serum starved (st) cells were treated for 2, 6 or 14 h with 10 µM *clasto*-lactacystin β-lactone (L), or for 6 or 14 h with 40 ng/ml HGF (H). Some cultured cells were pre-treated (2 h) with L and then stimulated with HGF (L/H) for 6 or 14 h. Nuclear proteins were prepared from treated cells and were used for EMSA after incubation with the labelled oligonucleotide probe corresponding to the HRE sequence. Inducible (HIF-1), constitutive (const) and unspecific (unsp) protein–DNA complexes are indicated. (**B**) Super gel-shift analysis of DNA binding complexes was performed using the same nuclear protein extracts as in (A) and the anti-HIF-1α antibody (OZ15). An asterisk indicates the position of the supershift complex. (**C**) Serum starved (st) cells were treated for 1 or 6 h with 5 mM *N*-acetylcysteine (N), 2 mM dithiotheritol (D) or 40 ng/ml HGF (H). Some cultured cells were pre-treated (1 h) with N or D, followed by stimulation with HGF (N/H or D/H) for 6 h. Nuclear proteins were prepared from treated cells and were used for EMSA after incubation with the labelled oligonucleotide probe corresponding to the HRE sequence. Inducible (HIF-1), constitutive (const) and unspecific (unsp) protein–DNA complexes are indicated. (**D**) Super gel-shift analysis of DNA binding complexes was performed using the same nuclear protein extracts as reported in (C) and anti-HIF-1α antibody (OZ15). An asterisk indicates the position of the supershift complex. The autoradiograms shown are representative of at least three different experiments.

slightly diminished during the following observation period. Densitometric values of the specific bands were normalized to those of 18 S rRNAs. Statistical analysis of the average fold increases relative to starved cells of three separate experiments was performed, and the means ± SE are reported at the bottom of each panel. The mean densitometric values of the Hox, uPAR and uPA bands corresponding to mRNA levels in starved cells were 49.6 ± 5.8, 19.0 ± 2.1 and 36.8 ± 4.2 (OD for area in mm^2), respectively. The regulation seems concerted, since the enhanced mRNA expression of the specific receptor for uPA seems to follow that of the ligand serine protease, achieving a coordinate mechanism of action.

Involvement of proteasome and redox state in HGF-mediated activation of HIF-1

HIF-1α is synthesized and quickly degraded by the ubiquitin–proteasome system under normoxic conditions (half-life of 5 min) (21). The involvement of this proteolytic system in HIF-1 DNA binding has scarcely been investigated. In further studies we examined HIF-1 DNA binding using nuclear extracts from HGF-treated cells in the presence of 10 µM *clasto*-lactacystin β-lactone, a potent and specific proteasome inhibitor

(37,38). As reported in Figure 3A, 2 h pre-treatment with *clasto*-lactacystin β-lactone almost completely prevented HIF-1 DNA binding activation observed 6 h after HGF. In super gel-shift experiments (Figure 3B), we confirmed the presence of HIF-1α in the inducible DNA binding complex 6 h after HGF, while only a very faint supershift complex was observed in 6 h HGF-treated cells undergoing proteasome blockade. It is noteworthy that the activity of the inducible complex was, however, unaffected by the proteasome blocking agent used alone.

Characterization of the binding complexes to HIF-1 DNA recognition site has been reported in the literature. In addition to the induced HIF-1 binding activity, EMSA with HRE shows a prominent, specific constitutive band. Most if not all of this constitutive factor is closely related or identical to the transcription factors ATF-1 and CREB-1 (39). After 2 h treatment with *clasto*-lactacystin β-lactone alone or 6 h treatment with HGF (as shown also in Figure 1A), we observed a marked increase in DNA binding of the constitutive complex, that might contain ATF-1/CREB-1. It has been reported that members of the ATF family are degraded by the proteasome (37), and

ATF-1 might be a crucial component of HIF-1 mediated hypoxia-inducible gene expression in mammalian cells (39). Studies are in progress to analyse the composition of the complexes that bind HRE in addition to that containing inducible HIF-1α.

The C-terminal half of HIF-1α contains two transactivation domains and their interaction with co-activators CBP, p300, SRC-1 and TIF2 is regulated by cellular O_2 concentration and redox state (40,41). To clarify the possible involvement of redox changes in HIF-1 activation by HGF, we analysed the effect of two reducing agents acting with different mechanisms. To this purpose, 5 mM N-acetylcysteine (42) or 2 mM dithiothreitol (43) were added to cultured cells 1 h before HGF. N-Acetylcysteine is a unique compound which acts by its own reducing power and by stimulating the synthesis of the major cellular reductant, i.e. reduced glutathione (GSH). Dithiothreitol is a thiol reducing compound widely used *in vitro*.

As reported in Figure 3C, the DNA binding activity of HIF-1 in 6 h HGF-treated cells seemed to be slightly increased by dithiothreitol, while N-acetylcysteine showed an inhibitory effect. These data were confirmed by super gel-shift experiments with the specific anti-HIF-1α antibody to verify the presence of the regulated α subunit in the HIF-1 complex (Figure 3D). Also in these experiments, 6 h treatment with N-acetylcysteine or dithiothreitol alone seemed to enhance the binding activity of the so-called constitutive complex, suggesting once again that the activity of transcription factors of other families are modulated under our experimental conditions and bind HRE sequence in addition to HIF-1α/β.

The DNA binding activity of ubiquitous Oct-1 was unaffected by any of the inhibitor or combined treatments performed (data not shown).

Time-courses of MAPK activities after HGF

The next step in the investigation of HGF-triggered signal transduction pathways deals with the evaluation of MAPK activities at various times after HGF treatment (Figure 4A). Phosphorylation and dephosphorylation activities have been suggested to be critical in signalling pathways leading to HIF-1 activation. However, the MAPK(s) involved in HIF-1α phosphorylation and then in transcription factor activity might be different *in vitro* and *in vivo* (31,44). JNK/SAPK and p38 MAPK activities were measured by evaluating the phosphorylation of GST–c-Jun and GST–ATF2 after western blot analysis and blotting with specific anti-phosphoSer or anti-phosphoThr antibodies, respectively. We observed that stress kinases were activated by HGF between 2 and 4 h though to a different degree. At these times, JNK/SAPK activity increased 9- and 10-fold, and p38 MAPK activity 15- and 40-fold relative to starved cells. Then the enzyme activities declined progressively. p42/44 MAPK, two closely related protein kinases that preferentially regulate cell growth and differentiation (45), were identified as the two bands of GST–Elk1 phosphorylation. Enzymatic activity rapidly increased about 5-fold at 0.5 h, and remained practically constant until 4 h, diminishing thereafter toward the value of starved cells. The two phosphorylation bands showed similar changes. Considering the observed time-courses of the MAPK activities, we may hypothesize a role of these enzymes in HGF-induced HIF-1 activation through phosphorylation of the α subunit. Further studies have been performed with the purpose of clarifying the involvement of these protein kinases in HIF-1
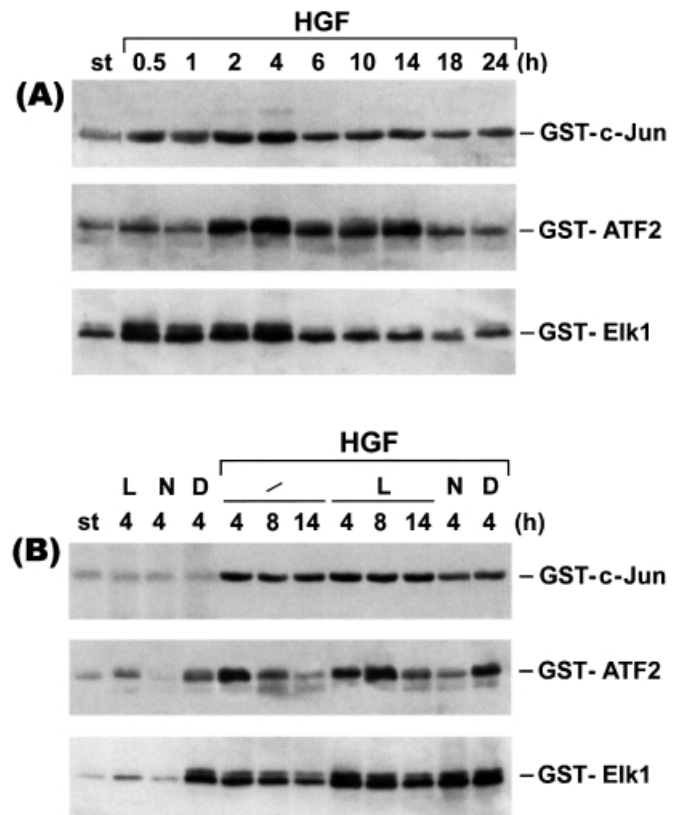
Fig. 4. Patterns of MAPK activities in response to HGF in cells undergoing proteasome blockade or treated with reducing agents. (**A**) Serum starved (st) cells were treated for various times with 40 ng/ml HGF and cell protein extracts were prepared to assess MAPK activities as reported in Materials and methods. GST–c-Jun (1–89), GST–ATF2 (19–96) and GST–Elk1 (307–428) were used as substrates to measure JNK/SAPK, p38 MAPK and p42/44 MAPK activities, respectively. Phosphorylation of the substrates was evaluated with western blot (in 10% SDS–PAGE) and blotting with anti-phospho-c-Jun (Ser63), anti-phospho-ATF-2 (Thr71) or anti-phospho-Elk-1 (Ser383) antibodies. (**B**) Serum starved (st) cells were treated for 4 h with 10 μM *clasto*-lactacystin β-lactone (L), 5 mM N-acetylcysteine (N) or 2 mM dithiotheritol (D). Some cultured cells pre-treated with L (2 h), N (1 h) or D (1 h) were then stimulated with 40 ng/ml HGF and used at the indicated times to measure MAPK activities, as reported in (A). The experiments have been repeated three times with similar results. The autoradiograms were evaluated by densitometric analysis. Statistical analysis of the data (OD/mm^2) was performed between the appropriate treatments and controls, i.e. HGF (0.5–24 h) versus st (A), or L/HGF, N/HGF and D/HGF versus HGF (B).

activity under our experimental conditions, and the findings are reported below.

Proposed mechanisms for the regulation of the MAPK cascade include proteasome activity and/or cell redox state (46,47). To understand whether these mechanisms might be involved in HGF-triggered MAPK activation, we studied the effect of pre-treatment with *clasto*-lactacystin β-lactone or with the reducing agents N-acetylcysteine and dithiothreitol. Figure 4B shows that *clasto*-lactacystin β-lactone enhanced the activities of p38 MAPK (about 3-fold) at 8 h and p42/44 MAPK (about 2-fold) between 4 and 8 h relative to HGF-treated cells and prevented their decrease thereafter. The activity of JNK was much less affected by *clasto*-lactacystin β-lactone in combination with HGF, increasing of about 1.5-fold at 8 h. Experiments with N-acetylcysteine and dithiothreitol were performed 4 h after HGF treatment when the maximal stimulation of the MAPK activities was observed.

HGF-induced activation of JNK and p38 MAPK was prevented by pre-treatment with *N*-acetylcysteine by 60 and 90%, respectively. Dithiothreitol showed an inhibitory effect lower than that of *N*-acetylcysteine. p42/44 MAPK activity stimulated by HGF was unaffected by both reducing agents. The inhibitors used alone did not modify appreciably the enzyme activities of starved cells at 4 h, except dithiothreitol that stimulated p38 and p42/44 MAPK activities (Figure 4B). Similar data were obtained at 8 and 14 h (data not shown).

PI3K cascade triggered by HGF

The PI3K pathway controls a number of cellular processes including cytoskeletal organization, cell growth and survival. PI3K activity is up-regulated in response to HGF and is known to be the key effector in the HGF-induced motility response (35,48). We next wanted to clarify the involvement of PI3K-triggered transduction pathway(s) in HIF-1 DNA binding and HIF-1α protein levels, as well as in the activation of p42/44 MAPK and JNK/SAPK, by using two specific inhibitors: LY 294002, that acts on the ATP binding site of PI3K, and PD 98059, that selectively blocks the activity of MAPK kinase (MEK 1/2) and, therefore, inhibits the activation of p42/44 MAPK (31,35). EMSA analysis (Figure 5A) shows that 30 min pre-treatment with 20 μM LY 294002 completely prevented the enhancement of HIF-1 DNA binding observed 6 h after HGF treatment, while 30 min pre-treatment with 100 μM PD 98059 was ineffective. These data were confirmed by super gel-shift analysis, since the shift of the inducible complex by OZ15 antibody did not occur when HGF was added to cells undergoing PI3K blockade (Figure 5B). Oct-1 DNA binding was unmodified by HGF treatment alone or in combination with the inhibitors (data not shown).

Consistent with these data, HIF-1α nuclear protein levels evaluated by western blot were enhanced by HGF in the presence or absence of PD 98059, while the combination of HGF plus LY 294002 prevented the increase in HIF-1α nuclear protein level (Figure 5C). Also the pre-treatment with *clasto*-lactacystin β-lactone prevented the increase in HIF-1α nuclear protein level due to HGF, in agreement with data from EMSA and super gel-shift assays (Figure 3A and B). Proteasome blockade actually increased cytosolic levels of the α subunit at 4 h after HGF treatment that declined slightly thereafter (14 h), suggesting a stabilization of HIF-1α protein even if it does not translocate in the nucleus (Figure 3D).

As reported in Figure 5E, PD 98059 actually inhibited p42/44 MAPK activity under our experimental conditions since it blocked enzyme activation 4 h after HGF, showing therefore values similar to those of starved cells. p42/44 MAPK activation by HGF was reduced of about 30% by LY 294002. The observed contribution of PI3K to the regulation of p42/44 MAPK is in agreement with the literature (35,49). Treatment with inhibitors alone for short time periods (30 min) diminished p42/44 MAPK activity in starved cells, and the effect was more evident in the case of PD 98059 than with LY 294002. Similar results were observed up to 6 h (data not shown). JNK/SAPK activation by HGF at 4 h was prevented by 50% in cells pre-treated with LY 294002, while PD 98059 had no effect on the enhancement of JNK/SAPK enzyme activity. These observations suggest that PI3K up-regulation after HGF treatment may be essential for the induction of the HIF-1α subunit. Indeed, the decrease in the amount of α subunit protein level might explain the reduction in HIF-1 DNA binding under PI3K blockade.
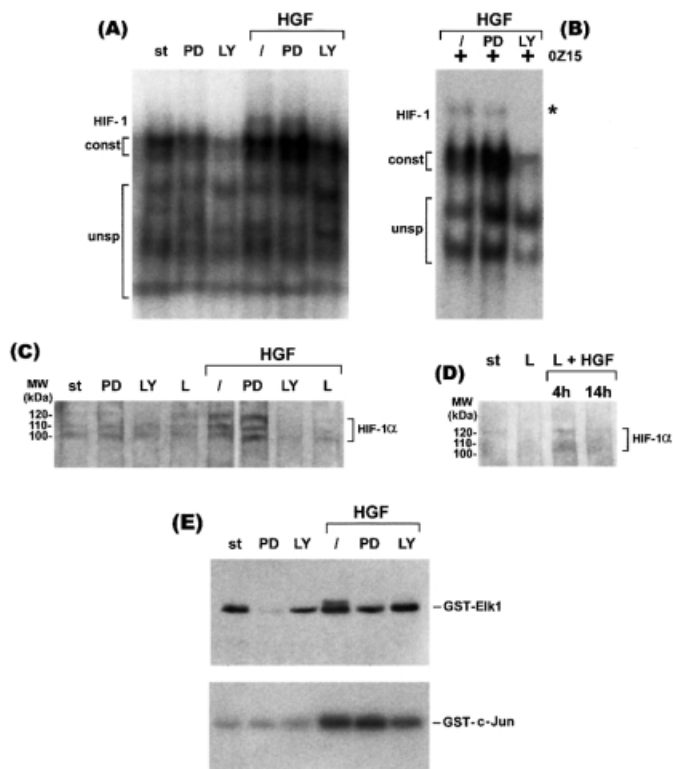
Fig. 5. PI3K involvement in HGF-induced HIF-1 activity and HIF-1α protein level. (**A**) Serum starved (st) cells were treated for 30 min with 100 μM PD 98059 (PD) or 20 μM LY 294002 (LY) and were then stimulated with 40 ng/ml HGF for 6 h. Nuclear proteins were prepared from treated cells and, after incubation with the labelled oligonucleotide probe corresponding to the HRE sequence, were used for EMSA. Inducible (HIF-1), constitutive (const) and unspecific (unsp) protein–DNA complexes are indicated. (**B**) Super gel-shift analysis of DNA binding complexes were performed using the same nuclear protein extracts as reported in (A) and the anti-HIF-1α antibody (OZ15). An asterisk indicates the position of the supershift complex. (**C**) Western blot of nuclear extracts prepared from cells treated with PD, LY or *clasto*-lactacystin β-lactone (L) in the presence or absence of 6 h HGF as reported above, separated by SDS–PAGE (7% gel) and blotted with anti HIF-1α antibody (clone H1α67). (**D**) Western blot of cytosolic extracts prepared from cells pre-treated with L for 2 h and then stimulated with HGF (4 or 14 h), separated by SDS–PAGE (7% gel) and blotted with anti HIF-1α antibody (clone H1α67). (**E**) Cells pre-treated with the inhibitors as reported in (A) and then exposed to HGF for 4 h. Total cell protein extracts were prepared to assess MAPK activities as reported in Materials and methods. GST–Elk1 (307–428) and GST–c-Jun (1–89) were used as substrates to measure p42/44 MAPK and JNK/SAPK, respectively. Phosphorylation of the substrates was evaluated by western blot (in 10% SDS–PAGE) and blotting with anti-phospho-Elk-1 (Ser383) or anti-phospho-c-Jun (Ser63) antibodies. Experiments were repeated three times with similar results. The autoradiograms were evaluated by densitometric analysis. For (E), the statistical analysis of the data (OD/mm^2) was performed between the appropriate treatments and control, i.e. PD/HGF and LY/HGF versus HGF (4 h).

Discussion

In this study, we demonstrate that HGF activates HIF-1 DNA binding and induces the expression of HIF-1 target genes Hox, uPAR and uPA. The 5′-flanking region of uPA gene shows numerous potential HREs, as reported also for uPAR (25). The 163 bp fragment located ~9.5 kb upstream of the transcription start site for Hox contains two binding sites for the HIF-1 transcription factor (36). HIF-1 transactivating activity controls tumour vascularization and metabolic adaptation to hypoxia, which are important steps in tumour progression and acquisition of the metastatic phenotype (21). Metastatic cells

additionally exhibit increased attachment to the extracellular matrix and proteolysis of basement membranes, enhanced migration and ability to colonize target organs. The pleiotropic effects of HGF might contribute to invasiveness and metastic growth also through the coordinate expression of the HIF-1 target genes that regulate activation and secretion of proteases, cell motility and angiogenesis. Our findings demonstrate a time-dependent regulation of uPA and uPAR messenger expression in liver neoplastic cells by HGF, and suggest a possible role of the uPA/uPAR system in the invasive phenotype acquired by hepatoblastoma cells under HGF treatment. It is noteworthy that this proteolytic mechanism is also important for autocrine and paracrine activation of HGF precursor monomer on the cell surface (50,51). Growth factor–receptor interactions result in signal transduction that leads to changes in transcription of the uPA gene, but the mechanisms resulting in the high expression of this gene in malignant tumour cells is poorly understood. Although the biological activity of uPA seems to be highly regulated at post-translational and transcriptional levels (12,52), post-transcriptional control has been suggested in neoplastic cells (27). Here we show that HGF caused a large increase in uPA mRNA followed by a very rapid down-regulation consistent with its reported short half-life of 1 h. This control mechanism depends on the presence in the 3′-untranslated region of two regulatory regions, one of which has an AU-rich element (ARE) (27).

In the context of HGF-regulated HIF-1 target genes, Hox, that is known to protect against tissue injury induced by endotoxin, oxidants and cytokines, has also been proposed to play cytotoxic roles. Like a 'Trojan horse' (53), Hox produces carbon monoxide that is likely to generate oxidants by mitochondria. Also hypoxia results in increased generation of reactive oxygen species (ROS) in the mitochondria, due to decreased activity of cytochrome oxidase, that would stabilize and activate the HIF-1 complex (21,24,54). Moreover, ROS are produced in a variety of cell types stimulated by a series of ligands, including cytokines as well as peptide growth factors acting through tyrosine kinase and G-protein-coupled receptors. The ligand-triggered mini oxidative burst may function as a second messenger system. One of the mechanisms proposed for ROS generation by cytokines and growth factors is the activation of small GTPase signal transducers (55). The downstream targets of ROS remain largely unknown, but regulation of the MAPK cascade through a Ras-dependent mechanism as well as direct regulation of transcription factors have been proposed (21). A ROS-dependent activation of HIF-1 DNA binding in HGF-treated cells might explain the similarity with the response of HIF-1 to hypoxia. Our observation that *N*-acetylcysteine prevented HGF-induced HIF-1 activity supports this hypothesis. Similarly, NF-κB activation by a wide variety of stressful stimuli can be blocked by *N*-acetylcysteine suggesting a role of ROS in this activation (56). A direct antioxidant buffering role as a potent radical scavenger for *N*-acetylcysteine may be proposed, since low doses of the compound as those used in the present paper do not seem to increase GSH levels and glutathione redox ratio (57). Under our experimental conditions, *N*-acetylcysteine probably uncouples the upstream signal transduction from GTPases to MAPKs. The thiol reagent dithiothreitol that readily permeates cell membranes and protects sulfydryl groups, did not affect negatively HGF-induced HIF-1 activity, but perhaps showed a further stimulatory effect probably by protecting

critical cysteine(s), as also reported for redox-regulated cysteine residues of NF-κB (58).

Regulatory mechanisms for HIF-1 activity other than redox modulation may include HIF-1α expression (mRNA and protein levels), phosphorylation, degradation and nuclear translocation (24). We observed that HGF increased steady-state mRNA and protein levels of HIF-1α concomitant with transcription factor activation. Whereas it is known that in HepG2 cultured cells cytokines (IL-1 and TNF-α) under normoxic and hypoxic conditions do not modify HIF-1α mRNA levels (22), the above reported cytokines induce mRNA expression in human synovial tissue (59).

HIF-1α is degraded in the proteasome after ubiquitination under normoxic conditions and is one of the most quickly degraded proteins found in mammals (21,24). In HGF-treated cells, we observed that specific and irreversible proteasome inhibition by a lactacystin derivative reduced almost completely HIF-1 DNA binding and nuclear protein level of the α subunit. This is in agreement with the failure of HIF-1α to accumulate in the nucleus of hypoxic cells in the presence of proteasome inhibitors (38). Although Salceda and Caro (60) observe that DNA binding of HIF-1 complex is induced under proteasome inhibition, a correlation with activation of HRE-dependent reporter gene expression does not occur. This suggests that a non-functional form of HIF-1α is accumulated in the cytosol and/or that additional regulatory steps to induce functional activity of HIF-1α are required.

In mammalian cells, at least six independent MAPK signalling units appear to function. Among them, the biochemical properties of three MAPKs, i.e. p42/44 MAPKs, also known as extracellular-signal regulated kinases (ERK 1/2), JNK/SAPK and p38 MAPK have been characterized in some detail (45). Unlike the p42/44 MAPK signalling pathway, the JNK and p38 MAPK transducers are not activated by mitogens but preferentially by pro-inflammatory cytokines and by diverse arrays of cellular stresses, including radiation, hydrogen peroxide, heat and osmotic shock and withdrawal of growth factors. We have demonstrated that stress kinases JNK and p38 MAPK as well as p42/44 MAPK were activated by HGF. However, only the increase in enzyme activity of the stress kinases was prevented by *N*-acetylcysteine, consistent with the inhibitory role exerted by this antioxidant on HIF-1 DNA binding. Moreover, since the blockade of MEK 1/2, that activates p42/44 MAPK, did not affect HGF-stimulated HIF-1 DNA binding, we conclude that this does not seem to be the principal transduction pathway for transcription factor activation. It has been reported that *in vitro* HIF-1α is phosphorylated by p42/44 MAPK and not by stress kinases, but p42/44 MAPK is not activated in cells exposed to hypoxia, indicating that this enzyme does not play a role in HIF-1α phosphorylation *in vivo*. In contrast to JNK, p42/44 MAPK (ERKs) are redox sensitive, but are not direct targets of ROS (55).

To evaluate a possible cooperation between ROS and growth factor signals, and by considering the fundamental role of PI3K signalling pathway in the transduction cascade triggered by HGF/Met, we investigated the effect of PI3K blockade on downstream molecular events (7,48,49). The observation that in HGF-treated cells LY 294002 reduced nuclear levels of the protein α subunit and therefore HIF-1 DNA binding indicates a main role of the PI3K cascade in HGF signal transduction to the nucleus and in the ultimate activation of HIF-1. JNK/SAPK activity and/or ROS probably produced by GTPases, along the transduction pathway(s) triggered by HGF/Met

binding, are also likely to play a role which is suggested but not proved by our present experiments. Studies are in progress to clarify the involvement of stress kinases in HIF-1 phosphorylation and transactivation activity for the expression of genes responsible for cell invasiveness.

Acknowledgements

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