Increased Sp1 phosphorylation as a mechanism of hepatocyte growth factor (HGF/SF)-induced vascular endothelial growth factor (VEGF/VPF) transcription

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Summary

Hepatocyte growth factor (HGF/SF)-induced expression of vascular endothelial growth factor (VEGF/VPF) has been implicated in paracrine amplification of angiogenesis, contributing to angiogenic responses during inflammation, wound healing, collateral formation and tumor growth. We have shown previously that HGF/SF-mediated VEGF/VPF expression by keratinocytes is primarily dependent on transcriptional activation, and we mapped the HGF/SF-responsive element to a GC-rich region between bp –88 and –65. Sp1-like factors bind to this element constitutively; however the VEGF/VPF promoter is transactivated by HGF/SF in the absence of induced binding activity. In experimental approaches to clarify molecular mechanisms of Sp1-dependent VEGF/VPF gene transcription, neither HGF/SF-dependent changes in nuclear expression nor in relative DNA binding activity of Sp family members to the indicated element were observed. Thus, HGF/SF was hypothesized to induce VEGF/VPF gene transcription via increased transactivation activity of Sp1 owing to biochemical modification. In immunoprecipitation studies, HGF/SF was found to increase the amount of serine-phosphorylated Sp1, revealing a likely mechanism of HGF/SF-induced VEGF/VPF expression, as phosphorylation may enhance the transcriptional activity of Sp1. The contribution of different signaling molecules to HGF/SF-induced VEGF/VPF transcription was demonstrated by the use of chemical inhibition, of expression of kinase-deficient signaling proteins, and by the use of antisense oligonucleotides. Herein, we provide evidence that PI 3-kinase, MEK1/2 and PKC-ζ play a significant role in HGF/SF-induced VEGF/VPF promoter activation. Together, our results elucidate a critical pathway of paracrine amplification of angiogenesis, suggesting that HGF/SF-induced Sp1 phosphorylation may activate VEGF/VPF promoter activity that requires the contribution of distinct signaling molecules.

Key words: Neovascularization, Endothelial growth factors, HGF/SF, VEGF/VPF, Transcription factors, Trans-activation, Signal transduction, Promoter regions

Introduction

Vascular endothelial growth factor (VEGF/VPF) is recognized as a predominant regulator of blood vessel growth in cancer, wound repair and in numerous ischemic and inflammatory diseases (reviewed by Carmeliet and Jain, 2000). VEGF/VPF expression is effectively induced by limited availability of oxygen and glucose, or may be strongly upregulated by different cytokines and growth factors (reviewed by Dvorak et al., 1995; Ferrara, 1999). More specifically, VEGF/VPF expression by keratinocytes and epithelial-derived tumor cells is profoundly increased by autocrine expression of transforming growth factor-α (TGF-α) and other ligands of the epidermal growth factor receptor (Detmar et al., 1994; Gille et al., 1997) or by mesenchymal-cell-derived hepatocyte growth factor/scatter factor (HGF/SF) (Gille et al., 1998; Wojta et al., 1999) and keratinocyte growth factor (Frank et al., 1995) in a paracrine fashion.

HGF/SF is known for its pro-angiogenic properties (Rosen and Goldberg, 1997) and is secreted by mesenchyme-derived cells, including fibroblasts and smooth muscle cells. HGF/SF mediates its effects through binding to the Met receptor tyrosine kinase, which is predominantly expressed on cells of epithelial origin, suggesting a key role for HGF/SF as a paracrine mediator of mesenchyme-epithelial interactions (reviewed by Birchmeier and Birchmeier, 1994; Rosen et al., 1994). On the basis of its ability to induce VEGF/VPF expression in different cell types (Clifford et al., 1998; Moriyama et al., 1998), and to potentiate its angiogenic effect via upregulation of VEGF/VPF in vivo (Van Belle et al., 1998), a model of an indirect paracrine amplification loop of angiogenesis has been proposed.

We have previously shown that HGF/SF-mediated VEGF/VPF expression by primary and immortalized keratinocytes, as well as epithelial-derived tumor cells, is primarily dependent on transcriptional activation, since VEGF/VPF mRNA stability as a major determinant of mRNA abundance remains unchanged after HGF/SF stimulation (Gille et al., 1998). The HGF/SF-responsive region has been mapped to a GC-box-containing region between bp –88 and –65 of the VEGF/VPF gene promoter, which is critical for both

constitutive and HGF/SF-induced transcriptional activity. Sp1-like factors constitutively bind to this element, although the VEGF/VPF promoter is transactivated by HGF/SF in the absence of induced binding activity. As HGF/SF functions as a potent inducer of VEGF/VPF expression in different cell types (Dong et al., 2001; Van Belle et al., 1998) and probably contributes to paracrine amplification of angiogenesis via VEGF/VPF induction, we set out studies to elucidate molecular mechanisms underlying HGF/SF-induced VEGF/VPF gene transcription.

Binding of Sp family members to the cluster of Sp1 sites in close proximity to the transcription start of the VEGF/VPF promoter has been implicated previously in constitutive (Shi et al., 2001) and growth-factor-induced VEGF/VPF gene expression (Finkenzeller et al., 1997; Gille et al., 1998). In addition, evidence has been provided that transrepression of VEGF/VPF promoter activity by both von Hippel-Lindau and p53 tumor suppressor gene product is mediated through Sp1 interaction via the indicated GC-rich response region (Mukhopadhyay et al., 1997; Zhang et al., 2000). Regulation of Sp1-dependent transcription may be conveyed by changes in DNA binding activity, by association with other transcription factors, by changes in Sp1 abundance or in transactivation activity owing to biochemical modification, such as phosphorylation (reviewed by Black et al., 2001; Philipsen and Suske, 1999). In addition, Sp1-dependent transcription could be effected by changes in relative binding activity of different Sp family members, as the respective transactivation activity of Sp1 and Sp3 may vary in a promoter- and cell-type-specific fashion. Pertinent to these findings, basal and Ras-induced Sp1-dependent VEGF/VPF promoter activity has been experimentally linked to interaction with and phosphorylation by the atypical protein kinase C (PKC) isoform PKC-ζ in renal cell carcinoma and fibrosarcoma cell lines (Pal et al., 1998; Pal et al., 2001). The significance of PKC-ζ as an intermediate signaling protein was substantiated in a recent study on stretch-induced VEGF/VPF mRNA expression by retinal pericytes and endothelial cells (Suzuma et al., 2002).

To determine mechanisms that mediate HGF/SF-induced Sp1 site-dependent VEGF/VPF gene transcription, we analyzed nuclear expression levels, binding activities, transactivating properties and phosphorylation states of Sp family members in HGF/SF-stimulated immortalized HaCaT keratinocytes. These studies did not reveal effects on either nuclear expression levels or on relative changes of Sp1 and Sp3 DNA-binding activity in response to HGF/SF. Since the family members Sp1 and Sp3 exert comparable functional effects on transcriptional activation of the VEGF/VPF promoter, induction of VEGF/VPF gene transcription may be conveyed via increased Sp1 transactivation activity, as HGF/SF is herein found to increase intracellular amounts of phosphorylated Sp1 in immunoprecipitation experiments.

Upon binding to its Met receptor tyrosine kinase, HGF/SF has been shown to concomitantly activate a number of distinct protein modules, including PI 3-kinase and the mitogen-activated protein kinase/extracellular signal-related kinase kinase (MEK) signaling cassette, which direct a genetic program that has been shown to facilitate cell proliferation and invasive growth (reviewed by Comoglio and Boccaccio, 2001; Stuart et al., 2000). By the use of pharmacological inhibition, of expression of kinase-deficient signaling molecules and by the use of antisense oligonucleotides targeting expression of protein signal transducers, we provide evidence that both MEK1/2 and PI 3-kinase contribute to HGF/SF-induced VEGF/VPF promoter activation and subsequent protein expression by HaCaT keratinocytes. We herein show that HGF/SF increases the intracellular amount of serine-phosphorylated Sp1, most probably by engaging PKC-ζ as an intermediate signaling protein. This novel observation may provide a rational mechanism of paracrine HGF/SF-induced upregulation of VEGF/VPF expression that significantly adds to the understanding of a pathway implicated in indirect amplification of angiogenesis.

Materials and Methods

Reagents

Human recombinant HGF/SF (used at 100 ng/ml) was purchased from R&D Systems (Wiesbaden, Germany). Chemical inhibitors were used at concentrations as indicated: PD 98059 (50 μM; inhibitor of MEK1, New England Biolabs, NEB, Frankfurt, Germany), wortmannin (100 nM; PI 3-kinase inhibitor), LY 294002 (10 μM; PI 3-kinase inhibitor), RO 31-8220 (2-20 μM; broad range PKC inhibitor, including atypical isoform PKC-ζ), bisindolylmaleimide I (1 μM; inhibitor with high selectivity for PKC-α, -β, -δ and -ε) and calphostin C (1 μM; inhibitor with high selectivity for conventional PKC; all from Calbiochem, Bad Soden, Germany). SuperFect Transfection Reagent was from Qiagen (Hilden, Germany), and Lipofectamine PLUS from Gibco Invitrogen Corp. (Karlsruhe, Germany). Antibodies detecting phosphorylated ERK1/2, ERK1/2, phosphorylated Akt (serine 473, threonine 308), Akt, PKC-δ and (pan) phospho-threonine were purchased from Cell Signaling Technology (Frankfurt, Germany). The phospho-serine monoclonal antibody sampler kit was purchased from Biomol (Hamburg, Germany). The Sp1 antibody (PEP-2; used in EMSA and western blot analyses), Sp3 antibody (D-20), PKC-ζ (C-20; used for immunoprecipitation) and actin antibody (H-196) were purchased from Santa Cruz (Heidelberg, Germany). The Sp1 antibody used for immunoprecipitation experiments was from Sigma Chemicals (Deisenhofen, Germany). Peroxidase-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG were purchased from Amersham Biosciences (Freiburg, Germany). The polyclonal antibody directed against the phosphorylated activation loop Thr^{410} of PKC-ζ was kindly provided by Alex Toker (Chou et al., 1998). The ECL-Plus western blotting detection system was obtained from Amersham. Protein G Sepharose 4 Fast Flow was purchased from Amersham Biosciences, Protein A/G PLUS-Agarose was from Santa Cruz. Proteinase and phosphatase inhibitors were used at concentrations as follows: antipain (2 μg/ml), aprotinin (2.2 μg/ml), pepstatin A (1 μg/ml), leupeptin (2 μg/ml, all from Sigma), dithiothreitiol (DTT, 1 mM), sodium vanadate (Na_3VO_4, 1 mM), phenylmethylsulfonyl fluoride (PMSF, 1 mM; all from AppliChem, Darmstadt, Germany). Poly[dI:dC] and dNTPs were purchased from Amersham. Klenow fragment was from NEB, and [α-^{32}P]-dCTP was obtained from Dupont NEN Life Science (Dreieich, Germany).

Cell lines and culture conditions

HaCaT cells (utilized between passages 45 and 65; provided by N. E. Fusenig; German Cancer Research Center, Division of Carcinogenesis and Differentiation, Heidelberg, Germany) (Boukamp et al., 1988) and A431 cells (American Type Culture Collection, Rockville, MD) were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% fetal bovine serum (Gibco), 2 mM L-

glutamine (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotericin B (all from Sigma). The embryonic *Drosophila* cell line SL2 (provided by G. Suske, Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Germany) was maintained at 25°C in Schneider's medium (Gibco), containing supplements as mentioned above.

VEGF/VPF protein quantification

For quantitative determination of human VEGF/VPF protein concentrations in cell culture supernatants, the Quantikine Human VEGF Immunoassay from R&D Systems was utilized. HaCaT cells were grown in 12-well plates to confluence and rendered quiescent by changing to serum-free DMEM media for 24 hours. Cells were incubated with respective pharmacological inhibitors and were subsequently stimulated by HGF/SF for indicated time periods. Experiments were performed in triplicate. Total cellular protein was harvested by addition of 5 M sodium hydroxide followed by a freeze/thaw cycle. Subsequently, protein concentration was determined using the DC Protein Standard Assay (Bio-Rad, Munich, Germany).

Plasmids and antisense oligonucleotides

The –88/+54 bp VEGF/VPF CAT construct has been described previously (Gille et al., 1997). Briefly, the indicated promoter fragment with flanking 5′-*Hin*dIII and 3′-*Xho*I enzyme restriction sites to facilitate directional cloning into the pCAT basic vector (Promega, Mannheim, Germany) was synthesized by PCR technique. The construct carrying a two nucleotide mutation within the Sp1 consensus sites (Sp1 mut) was generated identically, except that a primer was used that included the two nucleotide mutations. The constructs were confirmed by sequencing. The –88/+54 bp VEGF/VPF luciferase construct (Milanini et al., 1998), the CMV-promoter-driven expression vectors containing cDNAs encoding wild-type and dominant-negative (a lysine 281 to tryptophan point mutation in the catalytic site) forms of PKC-ζ (Bandyopadhyay et al., 1997), the Sp1 (pPacUSp1), Sp3 (pPacUSp3; under the control of the *Drosophila* actin 5C promoter) expression, and the corresponding parent (pPacUbx) plasmids (Hagen et al., 1994) have been described earlier. The utilized antisense oligonucleotides targeted against expression of PKC-α and PKC-ζ have been reported previously (Pal et al., 2001; Shih et al., 1999), and were synthesized as phosphorothioate-modified derivates (PKC-α: GGTCCTGCTGG-GCAT; PKC-ζ: ATGCCCAGCAGGACC; from MWG Biotech AG, Ebersberg, Germany).

Transient transfection and analysis of reporter gene expression

HaCaT cells (1.5×10^6, seeded in 60 mm dishes) were transfected with 5 μg of appropriate –88/+54 bp VEGF/VPF CAT reporter construct (wt/mut) using the SuperFect Transfection Reagent (Qiagen). 24 hours after transfection, control transfectants were left untreated (media change) and test transfectants were exposed to HGF/SF in the absence or presence of chemical inhibitors. 16 hours later, lysates were obtained by rapid freeze/thaw cycles. Quantitative determination of chloramphenicol acetyltransferase (CAT) expression was performed by colorimetric enzyme immunoassay (CAT ELISA, Roche Molecular Biochemicals, Mannheim, Germany). CAT expression was normalized to the activity of the co-transfected pSV-β-galactosidase control vector (1 μg, Promega). β-galactosidase activity was determined by ELISA using the Enzyme Assay System from Promega. Internal plasmid controls were not influenced by treatment with the different protein signaling inhibitors. SL2 cells (1.5×10^6, seeded in 60 mm dishes) were transfected with 5 μg –88/+54 bp VEGF/VPF CAT construct along with different combinations of expression vectors (Sp1, Sp3) or parent vector (Suske, 2000). After transfection, cells were propagated for 48 hours in medium only. Cells were lysed by rapid freeze/thaw cycles, and CAT activity was analyzed as described above. For co-expression experiments with wild-type and dominant-negative PKC-ζ expression plasmids, transfections were performed by electroporation (Gene Pulser II, Bio-Rad) using 1×10^6 HaCaT cells in the presence of 2.5 μg of the –88/+54 bp VEGF/VPF luciferase construct and 500 ng of expression vector. 24 hours after transfection, cells were stimulated with HGF/SF or were left untreated. Reporter gene activity was measured after 16 hours by the Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany), utilizing Promega's Luciferase Reporter Assay System. For inhibition of PKC isoform expression by antisense oligonucleotides, HaCaT cells (2×10^5, seeded in 12 well dishes) were transfected by Lipofectamine PLUS (Gibco) following the supplier's instructions with 1 μg of –88/+54 bp VEGF/VPF luciferase construct and 0.01 μM or 0.1 μM (final concentration) of the respective antisense oligonucleotides. 24 hours after transfection, cells were stimulated with HGF/SF for 16 hours or were left untreated. The activity of firefly luciferase was analyzed as described above.

Preparation of nuclear extracts and gel mobility shift analysis

HaCaT cells were treated with HGF/SF for 60 minutes. Nuclear proteins were extracted as described previously (Dignam et al., 1983). The oligonucleotides were synthesized to span the region between –88 bp and –65 bp of the human VEGF/VPF promoter: 5′-*TTT*CCGGGGCGGGCCGGGGGCGGGGTA*T*-3′. The underlined sequence served as a template for the synthesis of the second strand (random non-wild-type flanking sequences are shown in italic letters). Radiolabeled double-stranded DNA was synthesized by annealing an oligonucleotide complementary to the underlined sequence listed above (5′-ATACCCCGCCCC-3′) and by extension of the second strand with Klenow fragment, 50 μCi of [α-^{32}P]-dCTP, unlabeled dATP, dTTP and dGTP. Unincorporated nucleotides were removed by column chromatography. Cold unlabeled double-stranded DNA was made in the same way except that unlabeled dCTP was substituted for labeled dCTP. The DNA-binding reaction was performed for 30 minutes at room temperature in a volume of 20 μl, containing 5 μg of nuclear protein extract, 2.5 mg/ml bovine serum albumin, 10^5 cpm [α-^{32}P]-labeled probe (≈0.5-1.0 ng), 0.1 mg/ml poly[dI:dC] (Sigma), 5 μl of 4× binding buffer [1× buffer: 10 mM Tris-Cl, pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT] with or without excess of unlabeled competitor, Sp1 consensus-oligonucleotide (Promega), Sp1 or Sp3 antibody. Samples were subjected to electrophoresis on a native 4% polyacrylamide gel (PAGE) for 2.5 hours at 120 V.

Western blot analysis

Cells were grown to confluence and rendered quiescent by changing to serum-free media for 24 hours. Prior to HGF/SF exposure for 10 minutes, cells were treated with chemical inhibitors for 1 hour or were left untreated. Cells were washed with cold phosphate-buffered saline (PBS), lysed in sample buffer containing 187.5 mM Tris-HCl, pH 6.8, 6% sodium dodecyl sulphate (SDS) (w/v), 30% glycerol (v/v), 0.3 mM DTT, 0.3% bromophenol blue sodium salt (w/v), sonicated and boiled at 95°C for 5 minutes. Lysates were separated in 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF)-membrane (Millipore, Eschborn, Germany) at 50 V, subsequently blocked in Tris-buffered saline-Tween 20 (TBS-T) containing 5% BSA or non-fat milk. The membranes were incubated with primary antibodies followed by incubation with horseradish-peroxidase-conjugated secondary antibodies (anti-mouse or anti-rabbit, Amersham Biosciences). The blots were then developed using the enhanced chemiluminescence detection system (ECL) according to the instructions of the manufacturer (Amersham). For time-dependent

analysis of Sp1 and Sp3 protein, extracts were obtained by the procedure outlined for nuclear extracts.

Immunoprecipitation

For Sp1 immunoprecipitation, cells were lysed in cold buffer containing 20 mM Tris-HCl, 300 mM NaCl, 2 mM EDTA, 2 mM ethylenglycol-bis-(β-aminoethyl ether)-tetraacetic acid, 2% Triton (v/v), 1% NP-40 (v/v), supplemented with proteinase/phosphatase inhibitors, for 30 minutes on ice. Protein concentrations were determined with the DC Protein Standard Assay (Bio-Rad). Immunoprecipitations were carried out at antibody excess, incubating 2.5 mg of total lysate with of Sp1 antibody (Sigma) on a rotator at 4°C overnight. Immunocomplexes were then captured with Protein G Sepharose 4 Fast Flow (Amersham Biosciences). After three washes with lysis buffer, the immunoprecipitates were resuspended in electrophoresis sample buffer and subjected to western blot analysis. For PKC-ζ immunoprecipitation experiments, the protocol previously reported by Standaert et al. was used with minor modifications (Standaert et al., 1997).

Statistics

Normality (after Kolmogorov-Smirnov) of the data was confirmed by SigmaSTAT (SPSS Inc. Chicago). For statistical analysis, a student's *t*-test was performed using the Excel software from Microsoft (Redmond, WA). A $P<0.05$ on the basis of at least three independent sets of experiments was considered to be statistically significant.

Results

HGF/SF does not affect nuclear expression or relative DNA-binding activity of Sp family members

HGF/SF has been shown previously to mediate induced VEGF/VPF gene transcription through a GC-rich element at bp –88/-65 in the absence of enhanced DNA-binding activity (Gille et al., 1998). To determine molecular mechanisms that confer HGF/SF-induced Sp1 site-dependent VEGF/VPF gene transcription, we analyzed whether HGF/SF stimulation could affect relative binding of Sp family members as a consequence of alteration of nuclear levels of expression. Western blot analyses on extracts of untreated and HGF/SF-treated HaCaT cells, however, did not reveal a time-dependent regulation of nuclear expression of Sp1 and/or Sp3 (Fig. 1). Even at later time points (4, 8 and 16 hours), no changes in expression of Sp1 and Sp3 were observed (data not shown).

To clarify whether HGF/SF induced differences in relative DNA-binding activity of Sp family members to the cluster of Sp1 sites despite the absence of changes in nuclear expression levels, super-shift analyses were carried out using the –88/–65 bp promoter sequence as a specific DNA probe (Fig. 2). These electromobility shift assays (EMSAs) revealed multiple constitutive binding activities that were almost entirely Sp dependent, as shown by competition with excess Sp1 consensus oligonucleotides (lanes 3 and 4). Addition of antibodies directed against either Sp1 or Sp3 induced a supershift and/or a significant reduction of Sp1-dependent binding activities (lanes 5 to 8). Simultaneous addition of both antibodies leads to a nearly complete supershift (lanes 9 and 10). These data indicate that Sp1-dependent binding activity mostly comprises Sp1 and Sp3 protein, which is in line with previous reports on NIH3T3 fibroblasts (Finkenzeller et al., 1997) and pancreatic adenocarcinoma cell lines (Shi et al., 2001). However, both competition and super-shift analyses revealed no discernible differences in binding activities between extracts of untreated and HGF/SF-stimulated cells (Fig. 2). Thus our results are in opposition to the assumption that an induced change in relative DNA binding activity of Sp1 and/or Sp3 protein may operate as a mechanism by which HGF/SF activates VEGF/VPF gene transcription in the

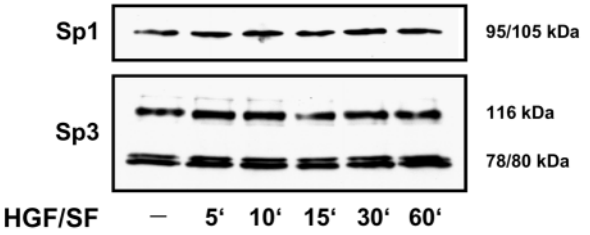

Fig. 1. HGF/SF does not regulate nuclear expression of Sp1 and Sp3 transcription factor in HaCaT cells. Representative western blot analysis of nuclear Sp1 and Sp3 expression by quiescent HaCaT cells, either left untreated (media change) or stimulated by HGF/SF for the indicated time periods. Total cellular protein (10 μg/lane) was separated by 10% SDS-PAGE, Sp1 and Sp3 protein were detected by enhanced chemiluminescence using specific antibodies to the respective nuclear factors (Santa Cruz; molecular weights are indicated to the right). Comparable results were obtained from three independent experiments.

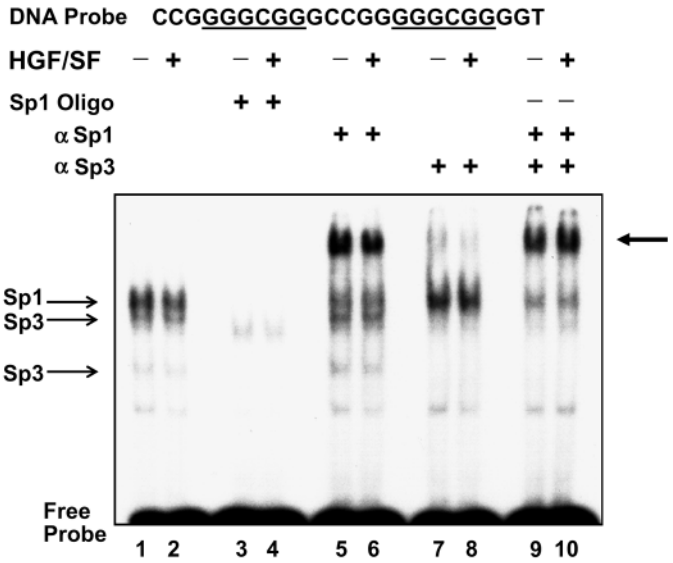

Fig. 2. HGF/SF neither changes relative DNA-binding activity of Sp1 and Sp3 factor nor induces additional Sp1-indendent binding to the –88/-65 bp VEGF/VPF promoter sequence. A representative EMSA using nuclear extracts of untreated (lanes 1, 3, 5, 7 and 9) or HGF/SF-stimulated HaCaT cells (10 minutes; lanes 2, 4, 6, 8 and 10). Unlabeled Sp1 consensus oligonucleotides (Promega) were used at a final concentration of 0.35 μM (lanes 3 and 4). Supershift analysis was performed by addition of specific Sp1 or/and Sp3 antibodies (Santa Cruz) at a final concentration of 100 ng/μl (lanes 5 to 10). The DNA sequence of the utilized probe is shown at the top (Sp1 sites underlined), the formation of Sp1-dependent binding complexes is indicated by arrows to the left; supershifted complexes are marked by a bold arrow to the right.

absence of inducible DNA-binding activity to the functional response element.

Both Sp1 and Sp3 transcription factor induce Sp1 site-dependent VEGF/VPF promoter activation in a similar fashion to *Drosophila* SL2 cells

Extending our DNA-binding experiments, we subsequently determined whether the HGF/SF effect could be mediated through differing transactivating properties of the respective Sp family members as a function of subtle binding variations to the GC-boxes. We therefore explored whether Sp1 and Sp3 exerted different effects on VEGF/VPF promoter activity and whether their individual and/or combined effects were preferentially mediated via the Sp1 consensus binding sites. To eliminate the impact of constitutive expression of Sp family members in HaCaT keratinocytes, these experiments were performed in *Drosophila* Schneider SL2 cells. As the HGF/SF-response has been functionally mapped to a VEGF/VPF promoter region between bp –88 and –70 (Gille et al., 1998), the expression vectors encoding Sp1 and Sp3 were co-transfected with the –88/+54 bp VEGF/VPF reporter plasmid. Initially, the dosage of maximal reporter gene induction by the respective expression plasmids was determined. The results were similar with both plasmids, and maximal induction was seen with 250 ng to 500 ng of co-transfected DNA, with no further increase observed with higher amounts (data not shown). Co-expression of either Sp1 or Sp3 revealed marked activation of VEGF/VPF promoter activity compared with cells co-transfected with backbone vector only (Fig. 3). Sp1 expression only tended to act as a slightly more effective inducer of transcriptional activation than Sp3; however, the differences were not seen to be statistically significant. In addition, simultaneous expression of Sp3 and Sp1 did not attenuate Sp1-driven reporter gene activity, as has been observed previously with different promoters (Hagen et al., 1994). In order to demonstrate that induced expression by Sp1 and Sp3 was Sp1 site dependent, a –88/+54 bp VEGF/VPF reporter plasmid was utilized that carried two critical nucleotide mutations within the adjacent Sp1 sites. Indeed, co-expression experiments with this mutant construct showed marked inhibition compared to co-transfections with the respective wild-type plasmid. Together, these results indicate that HGF/SF-induced Sp1 site-dependent VEGF/VPF transcription may not be mediated by relative changes of Sp1 and/or Sp3 DNA binding or by differing transactivating properties of the respective Sp family members, taking into account that subtle binding variations to the GC-boxes may not have been detected by EMSA.

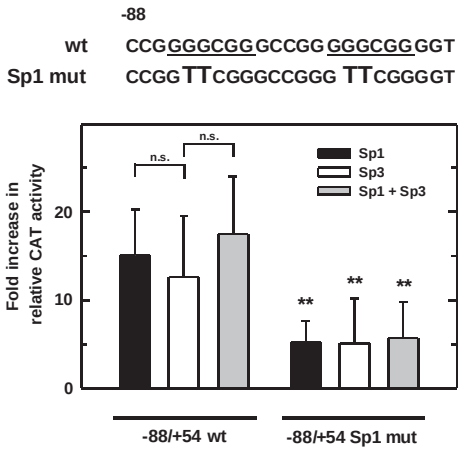

Fig. 3. Sp1 and Sp3 transcription factor confer Sp1-site dependent VEGF/VPF promoter transactivation in a comparable fashion in *Drosophila* SL2 cells. Analysis of CAT expression derived from transiently transfected –88/+54 bp wild-type (wt) or –88/+54 Sp1 site-mutated (Sp1 mut) VEGF/VPF promoter-based construct (carrying critical 2 nucleotide mutations within the Sp1 sites; 5 μg each) along with expression vectors encoding Sp1 (pPacUSp1, 0.3 μg), Sp3 (pPacUSp3, 0.3 μg) and/or empty vector (pPacUbx, to compensate for differences in co-transfected DNA). The fold increase in CAT activity was calculated on the basis of data obtained from cells co-transfected with empty vector alone. Schematic representations of the wild-type and the mutated sequence are shown at the top (Sp1 sites underlined), the two nucleotide mutations are indicated by enlarged letter size. The data displayed represent the means±s.d. of five independent duplicate experiments. (Student's *t*-test; n.s., not significant; ** $P<0.01$, compared to cells transfected with the wt vector).

HGF/SF mediates serine phosphorylation of Sp1 transcription factor

In the absence of both apparent relative changes in Sp1 and Sp3 DNA-binding activity and significant functional differences in Sp family members, we proposed that HGF/SF increases transactivation activity of Sp1 as a molecular mechanism to induce VEGF/VPF gene transcription. As phosphorylation has been implicated in changes of Sp1 transcriptional activity (Alroy et al., 1999; Chun et al., 1998; Fojas et al., 2001), we next determined whether HGF/SF increased the amount of phosphorylated Sp1 (Fig. 4). For this purpose, whole protein extracts of untreated and HGF/SF-stimulated cells were immunoprecipitated by Sp1 antibody (Sigma) and were subsequently subjected to western blot analyses utilizing a panel of antibodies, specific for phospho-threonine-containing proteins (binding to threonine-

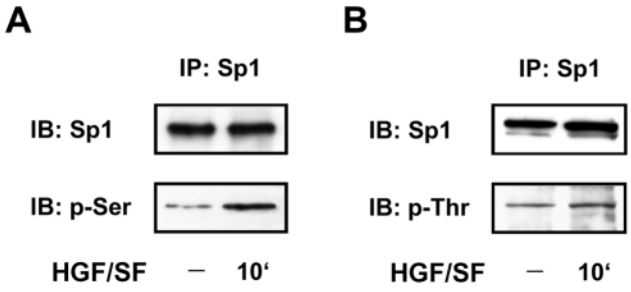

Fig. 4. HGF/SF increases cellular amounts of serine-phosphorylated transcription factor Sp1. Western blot analyses of HaCaT cells that were left untreated (media change) or were stimulated by HGF/SF (10 minutes at 100 ng/ml). Cellular extracts were immunoprecipitated (IP) by a specific anti-Sp1 antibody (Sigma) prior to immunoblotting (IB). (A) Immunoblotting with Sp1 antibody (upper panel) and phospho-serine monoclonal antibody (clone 4A3; lower panel). (B) Immunoblotting with Sp1 antibody (upper panel) and phospho-threonine antibody (lower panel). The immunoblots displayed are representative of three that were performed revealing comparable results.

phosphorylated sites in a manner largely independent of the surrounding amino-acid sequence) or recognizing a distinct pattern of serine-phosphorylated proteins (preferring positively charged amino acids adjacent to phospho-serine). Among the different clones directed against phospho-serine-containing proteins (1C8, 4A3, 4A9, 4H4, 16B4, 7F12), the antibody designated 4A3 reproducibly detected a strongly enhanced signal in extracts of HGF/SF-treated cells compared with unstimulated controls (Fig. 4A). This effect was not HaCaT-cell-specific, as an HGF/SF-mediated increase in serine phosphorylated Sp1 was also observed in A431 carcinoma cells (data not shown). To monitor the correct level of migration, the blots were also probed with Sp1 antibody (Fig. 4A,B; upper panel). The remaining antibodies, including the one that recognizes phospho-threonine-containing proteins (Fig. 4B), did not display enhanced signal strength after HGF/SF treatment relative to untreated controls. These data indicate that HGF/SF is capable of increasing relative amounts of serine-phosphorylated Sp1 transcription factor in HaCaT keratinocytes. Nevertheless, phosphorylation events could involve threonine residues of Sp1 protein as well, which may have not been detected by the antibodies used. Together, in the absence of any changes regarding nuclear expression and DNA binding, the HGF/SF-induced increase in phosphorylated Sp1 may represent a rational mechanism to enhance Sp1's transcriptional activity, conveying HGF/SF-induced VEGF/VPF transcription.

HGF/SF-mediated transactivation of the VEGF/VPF promoter is repressed by atypical PKC-ζ antisense oligonucleotides and overexpression of dominant-negative PKC-ζ mutant, whereas inhibition of conventional/novel PKC isoforms fails to block induced VEGF/VPF transcription

HGF/SF has been demonstrated to transduce its biological activities through the Met receptor tyrosine kinase, activating a number of intracellular pathways to integrate the HGF/SF signal to the cytosol and to the nucleus (reviewed by Comoglio and Boccaccio, 2001; Stuart et al., 2000). Responses to HGF/SF binding have been shown to involve activation of different functional protein kinase C (PKC) subspecies that are associated with enhanced cell migration and growth (Cai et al., 2000; Chandrasekher et al., 2001) and are linked in part to increased invasive potential of cancer cells owing to induction of protease expression (Kermorgant et al., 2001). As classical and novel PKC activation contributes to induced VEGF/VPF expression in response to certain stimuli (Hossain et al., 2000; Kim et al., 2000), we sought to determine the potential contribution of PKC activation to the HGF/SF-induced VEGF/VPF gene expression. To verify whether HGF/SF activates conventional/novel PKC isoforms in HaCaT keratinocytes, western blot analyses of extracts from treated and unstimulated cells were performed (Fig. 5A), utilizing an antibody that detects the phosphorylated PKC-δ isoform.

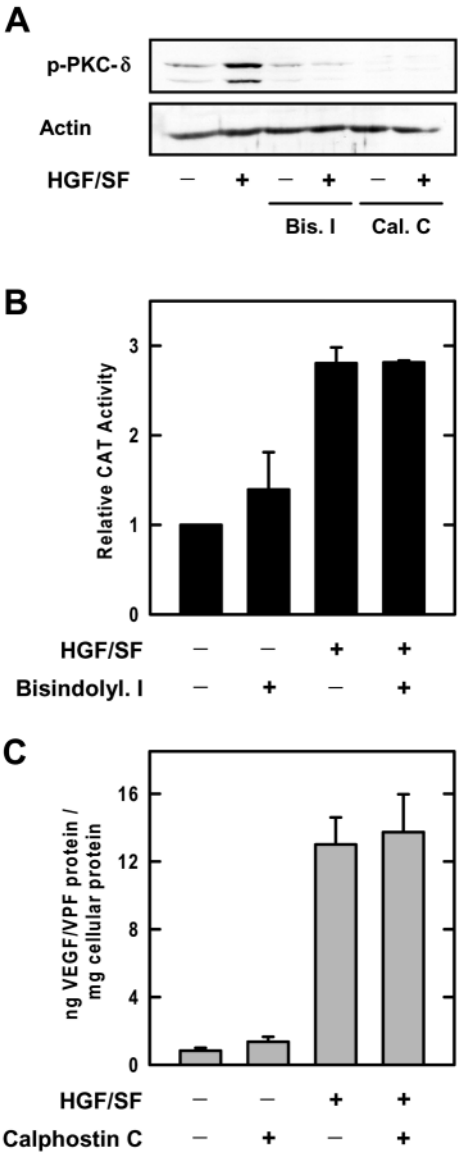

Fig. 5. Pharmacological inhibition of conventional and novel PKC isoforms blocks HGF/SF-induced PKC phosphorylation but fails to inhibit HGF/SF-mediated VEGF/VPF gene expression. (A) Detection of phosphorylated PKC-δ (p-PKC-δ, upper panel) or the respective actin protein expression (from Santa Cruz) by western blot analysis. HaCaT cells were left untreated or were exposed to HGF/SF (for 10 minutes at 100 ng/ml) after preincubation with broad-range PKC inhibitor calphostin C (Cal. C at 1 μM for 60 minutes), isotype-selective (conventional and novel) PKC inhibitor bisindolylmaleimide I (Bis. I at 1 μM for 60 minutes) or solvent only (DMSO, 0.1%) as indicated. Experiments were repeated twice with similar results. (B) Analysis of CAT expression derived from a transiently transfected –88/+54 bp VEGF/VPF-promoter-based construct. HaCaT cells were cultured in the absence or presence of bisindolylmaleimide I (Bis. I at 1 μM, starting 1 hour prior to HGF/SF treatment) without growth factor stimulation or with HGF/SF treatment (for 16 hours at 100 ng/ml) as indicated. Data displayed represent the means±s.d. of three independent triplicate assays. (C) VEGF/VPF protein of supernatants derived from confluent HaCaT cells. Cells were cultured in the absence or presence of calphostin C (at 1 μM, starting 1 hour prior to HGF/SF treatment) without growth factor stimulation or with HGF/SF treatment (for 24 hours at 100 ng/ml) as indicated. Data from three independent triplicate experiments are expressed as ng secreted VEGF/VPF protein per mg total cellular protein (mean±s.e.m.).

HGF/SF was seen to increase PKC phosphorylation, which was substantially diminished by preincubation with known conventional/novel PKC inhibitors (bisindolylmaleimide I, calphostin C). Consequently, we clarified whether pharmacological PKC inhibition blocked HGF/SF-induced VEGF/VPF promoter activation (Fig. 5B). Both bisindolylmaleimide I and calphostin C (data not shown) failed to abrogate VEGF/VPF-promoter-based reporter gene expression, however (Fig. 5B). These observations are in line with effects observed on induced VEGF/VPF protein expression by HaCaT keratinocytes, as chemical PKC inhibition failed to block protein synthesis as well (Fig. 5C, equivalent data were obtained from studies with bisindolylmaleimide I). This data thus indicate that HGF/SF-induced VEGF/VPF expression by HaCaT keratinocytes is independent of conventional and novel PKC isoforms.

Besides conventional and novel PKC isoforms, atypical PKC-ζ has recently been closely linked to transcriptional activation of the VEGF/VPF gene (Pal et al., 1998; Shih et al., 1999). In our studies on HaCaT keratinocytes, PKC-ζ was promptly phosphorylated in response to HGF/SF (Fig. 6A). In the current absence of isotype-specific PKC-ζ inhibitors, explicit antisense oligonucleotides (AS) were utilized in transcriptional activation studies that have been previously shown to effectively block PKC isoform expression (Pal et al., 2001; Shih et al., 1999) (Fig. 6B). Whereas both PKC-ζ and PKC-α antisense oligonucleotides at 0.01 μM did not effect transcription (data not shown), at a concentration of 0.1 μM AS-PKC-ζ, but not AS-PKC-α, inhibited reporter gene expression. These observations are in agreement with our chemical inhibition studies (Fig. 5B), revealing a lack of inhibitory efficacy on VEGF/VPF promoter activity by conventional/novel PKC inhibitor bisindolylmaleimide I. The potential contribution of PKC-ζ was substantiated in experiments in which expression of wild-type and dominant-negative PKC-ζ protein on VEGF/VPF gene transcription was analyzed (Fig. 6C). Overexpression of deficient PKC-ζ protein significantly inhibited basal and HGF/SF-induced transactivation. In addition, broad-range PKC inhibitor RO 31-8220, which also affects atypical PKC-ζ (Standaert et al., 1997), blocks HGF/SF-induced VEGF/VPF transcription in a concentration-dependent fashion (data not shown). Together, these findings suggest that PKC-ζ is engaged as an intermediate signaling protein in HGF/SF-induced VEGF/VPF expression.

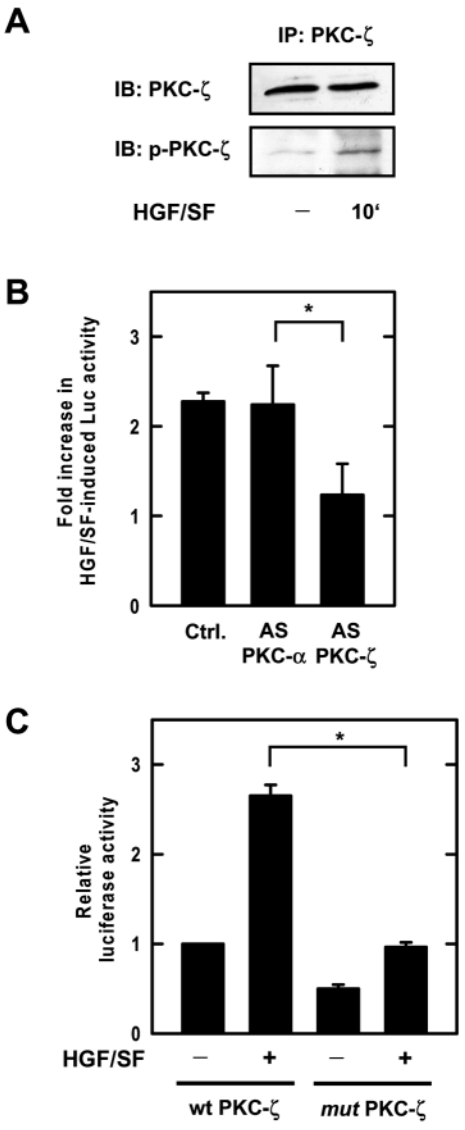

Fig. 6. Targeting PKC-ζ expression attenuates HGF/SF-induced VEGF/VPF promoter activity. (A) Western blot analyses of HaCaT cells that were left untreated (media change) or were stimulated by HGF/SF (10 minutes at 100 ng/ml). Cellular extracts were immunoprecipitated (IP) by a specific anti-PKC-ζ antibody (Santa Cruz) prior to immunoblotting (IB). Immunoblotting with pan-PKC-ζ antibody (Santa Cruz; upper panel) and with phospho-PKC-ζ antibody (lower panel). (B) Analysis of firefly luciferase (Luc) expression derived from a transiently transfected –88/+54 bp VEGF/VPF-promoter-based reporter construct (1 μg) along with antisense oligonucleotides directed against the translation start site of PKC-α (second bar) or of PKC-ζ (third bar, each at 0.1 μM). Twenty-four hours after transfection, HaCaT cells were stimulated with HGF/SF (at 100 ng/ml) for 16 hours or were left untreated. This assay is representative of three independent sets of experiments revealing comparable results. Fold increase in HGF/SF-induced luciferase activity is calculated on the basis of data obtained from the respective controls, which were left unstimulated. Values represent the mean±s.d. of triplicate assays. Statistical analyses were performed on data from three experiments (Student's *t*-test, **P*<0.05). (C) Analysis of firefly luciferase expression derived from a transiently transfected –88/+54 bp VEGF/VPF-promoter-based reporter construct (2.5 μg) along with wild-type PKC-ζ (wt-PKC-ζ) or its kinase-deficient mutant (mut-PKC-ζ; 500 ng each) vector. Twenty-four hours after transfection, HaCaT cells were stimulated with HGF/SF (at 100 ng/ml) for 16 hours or were left untreated. Data displayed herein include the values of three independent duplicate experiments (mean±s.e.m.; Student's *t*-test, **P*<0.05).

HGF/SF-induced VEGF/VPF gene expression is mediated via activation of PI 3-kinase and MEK1/2 signaling modules

Among the protein modules activated via HGF/SF binding to the Met receptor, PI 3-kinase and the MEK1/2 signaling cascades appear to act as transducers of central importance (Stuart et al., 2000). To determine whether additional signaling proteins are implicated in HGF/SF-mediated

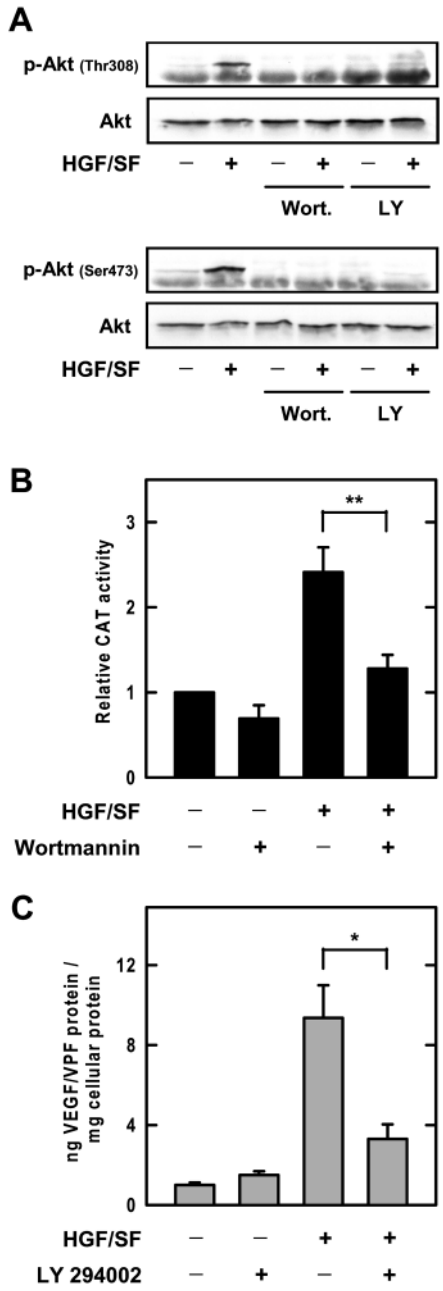

Fig. 7. Chemical inhibition of the PI 3-kinase pathway blocks HGF/SF-induced Akt phosphorylation and inhibits HGF/SF-mediated VEGF/VPF gene transcription and protein expression. (A) Detection of phosphorylated Akt (at residue threonine 308, Thr^{308}; upper panel; at serine residue 473, Ser^{473}, lower panel) or of the respective total Akt protein expression (Akt; antibodies from Cell Signaling) by western blot analysis. HaCaT cells were left untreated or were exposed to HGF/SF (for 10 minutes at 100 ng/ml) after preincubation with the PI 3-kinase inhibitors wortmannin (Wort., at 100 nM, for 60 min) or LY 294002 (LY; at 10 μM, for 60 min) or with solvent only (DMSO, 0.1%) as indicated. Experiments were repeated three times with comparable results. (B) Analysis of CAT expression derived from a transiently transfected –88/+54 bp VEGF/VPF-promoter-based construct. HaCaT cells were cultured in the absence or presence of wortmannin (at 100 nM, starting 1 hour prior to HGF/SF treatment) without growth factor stimulation or with HGF/SF treatment (for 16 hour at 100 ng/ml) as indicated. Values represent the mean±s.d. of three triplicate assays. (C) VEGF/VPF protein of supernatants derived from confluent HaCaT cells. Cells were cultured in the absence or presence of LY 294002 (at 10 μM, for 60 minutes, starting 1 hour prior to HGF/SF treatment) without growth factor stimulation or with HGF/SF treatment (for 24 hours at 100 ng/ml) as indicated. Data from three triplicate experiments are expressed as ng secreted VEGF/VPF protein per mg total cellular protein (mean±s.e.m.). Statistical analyses were performed on data from three sets of experiments (Student's *t* test, $^{**}P<0.01$, $^{*}P<0.05$).

VEGF/VPF transcription and protein expression by HaCaT cells, we evaluated the contribution of the indicated pathways largely by use of selective chemical inhibitors. HGF/SF activates Akt by phosphorylation at both the threonine 308 and the serine 473 residues (Fig. 7A). Abrogation of Akt activation in response to HGF/SF by the PI 3-kinase inhibitors wortmannin and LY 294002 clearly suggests that Akt phosphorylation in HaCaT keratinocytes is PI 3-kinase dependent. Moreover, both PI 3-kinase inhibitors effectively inhibit HGF/SF-induced VEGF/VPF-promoter-based reporter gene expression and subsequent protein expression (Fig. 7B,C), strongly implicating PI 3-kinase in the signaling pathway of HGF/SF-mediated VEGF/VPF expression.

In addition, selective inhibition of MEK1 by compound PD 98059 completely blocked HGF/SF-induced phosphorylation of downstream ERK1/2 (Fig. 8A), attenuated baseline VEGF/VPF expression and entirely prevented upregulation of VEGF/VPF gene transcriptional activation and protein expression (Fig. 8B,C). Equivalent results were obtained by utilizing the MEK1/2 inhibitor U0126 (data not shown), establishing a key contribution of the MEK1/2 signaling protein to HGF/SF-induced gene expression by HaCaT keratinocytes. As both PI 3-kinase and MEK1/2 inhibition resulted in abrogation of induced VEGF/VPF expression, the question arose of whether crosstalk between the indicated signaling pathways exists (Fig. 9A). As shown above, both PD 98059 and LY 294002 as explicit inhibitors blocked phosphorylation of their appropriate downstream targets in HaCaT cells; however, they failed to inhibit HGF/SF-induced activation of the respective mutual downstream signaling proteins. Whereas LY 294002 inhibited HGF/SF-induced Akt activation, it did not abolish ERK1/2 phosphorylation. Similarly, PD 98059 inhibited HGF/SF-induced MEK1/2 activation but revealed no effect on Akt activation (Fig. 9A). From a hierarchical point of view, PI 3-kinase and MEK1/2 thus appear to mediate the HGF/SF signal partly in parallel in HaCaT keratinocytes, as HGF/SF-induced activation of immediate PI 3-kinase and MEK1/2 downstream targets is mutually independent.

HGF/SF-induced phosphorylation of PKC-ζ and Sp1 is blocked by PI 3-kinase and MEK1/2 inhibition

Both pharmacological inhibitors of PI 3-kinase and MEK1 seem to jointly block shared downstream signaling proteins, as each inhibitor by itself almost completely prevents HGF/SF-mediated VEGF/VPF transcription and protein expression (Figs 7 and 8). Induced phosphorylation of respective downstream targets of PI 3-kinase and MEK1/2 occurs despite

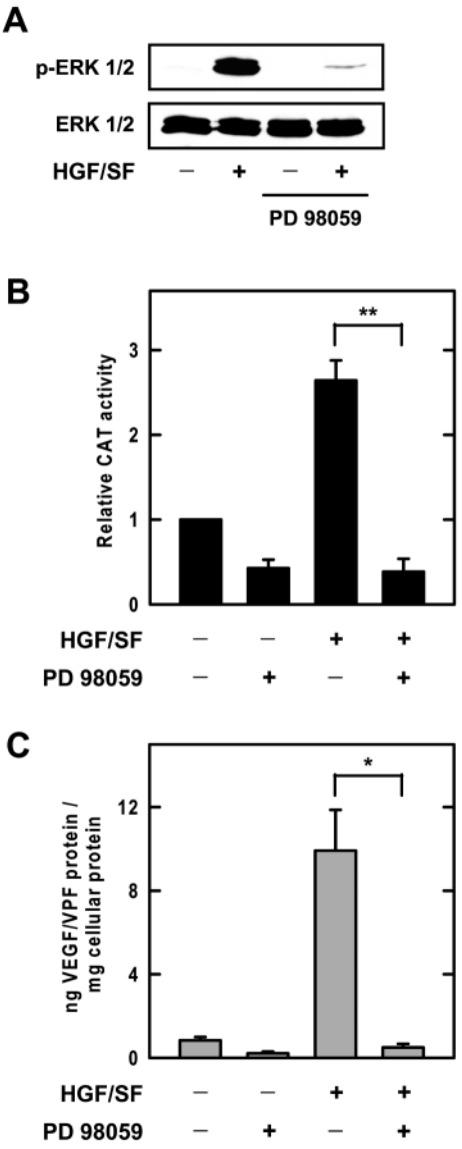

Fig. 8. Inhibition of MEK1/2 blocks HGF/SF-induced PKC phosphorylation of ERK1/2 and inhibits HGF/SF-mediated VEGF/VPF gene transcription and protein expression. (A) Detection of phosphorylated ERK1/2 (p-ERK1/2, upper panel) or total ERK1/2 protein (ERK1/2, lower panel) by western blot analysis. HaCaT cells were left untreated or were exposed to HGF/SF (for 10 minutes at 100 ng/ml) after preincubation with the MEK1/2 inhibitor PD 98059 (at 50 μM for 60 minutes) or solvent only (DMSO, 0.1%) as indicated. Experiments were repeated three times with comparable results. (B) Analysis of CAT expression derived from a transiently transfected –88/+54 bp VEGF/VPF-promoter-based construct. HaCaT cells were cultured in the absence or presence of PD 98059 (at 50 μM, starting 1 hour prior to HGF/SF treatment) without growth factor stimulation or with HGF/SF treatment (for 16 hours at 100 ng/ml) as indicated. The data displayed represent the mean±s.d. of three triplicate assays. (C) VEGF/VPF protein of supernatants derived from confluent HaCaT cells. Cells were cultured in the absence or presence of PD 98059 (at 50 μM, starting 1 hour prior to HGF/SF treatment) without growth factor stimulation or with HGF/SF treatment (for 24 hours at 100 ng/ml) as indicated. Data from three triplicate experiments are expressed as ng secreted VEGF/VPF protein per mg total cellular protein (mean±s.e.m.). Statistical analyses were performed on data from three sets of experiments (Student's *t*-test, $^{**}P<0.01$, $^{*}P<0.05$).

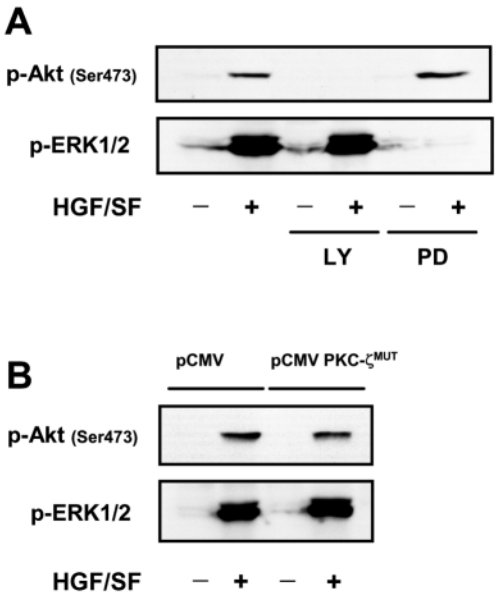

Fig. 9. Effects of MEK1 (PD 98059) or PI 3-kinase (LY 294002) inhibition on HGF/SF-induced phosphorylation of Akt and ERK1/2 in HaCaT keratinocytes. (A) Detection of phosphorylated Akt (at residue serine 473, Ser^{473}, upper panel) or detection of phosphorylated ERK1/2 (p-ERK1/2, lower panel, antibodies from Cell Signaling) by western blot analysis. HaCaT cells were left untreated or were exposed to HGF/SF (for 10 minutes at 100 ng/ml) after preincubation with PI 3-kinase inhibitor LY 294002 (LY; at 10 μM for 60 minutes), with the MEK1 inhibitor PD 98059 (PD; at 50 μM for 60 minutes) or with solvent only (DMSO, 0.1%) as indicated. (B) Detection of phosphorylated Akt or ERK1/2 in HaCaT cells that were either transiently transfected with parent vector only (pCMV; lanes 1 and 2) or with dominant-negative PKC-ζ mutant (pCMV PKC-$ζ^{MUT}$; 500 ng each). Thirty-eight hours after transfection, cells were left untreated or were exposed to HGF/SF (for 10 minutes at 100 ng/ml). Experiments were repeated twice with comparable results.

the presence of overexpressed kinase-deficient PKC-ζ (Fig. 9B), indicating that PKC-ζ may serve as a downstream effector of MEK1/2 and PI 3-kinase upon HGF/SF stimulation. This assumption is further supported by experimental evidence demonstrating that HGF/SF-induced activation of PKC-ζ is efficiently blocked by both PI 3-kinase and MEK1/2 inhibition (Fig. 10A,B). These data strongly imply that PI 3-kinase and MEK1/2 act as upstream signaling proteins in HGF/SF-induced PKC-ζ phosphorylation. To substantiate the importance of Sp1 phosphorylation in HGF/SF-induced VEGF/VPF gene expression, we next determined the effect of PI 3-kinase and MEK1/2 inhibition on HGF/SF-induced Sp1 phosphorylation. In agreement with our hypothesized model, induced phosphorylation of Sp1 is prevented by pharmacological inhibition of PI 3-kinase and MEK1 (Fig. 11). In addition, broad range PKC inhibition by compound RO 31-8220 effectively blocked HGF/SF-induced Sp1 phosphorylation, suggesting that Sp1 may function as a downstream target for PI 3-kinase, MEK1/2 and PKC-ζ. Together, these data indicate that HGF/SF induces serine-phosphorylation of Sp1 transcription factor via PI 3-kinase, MEK1/2 and PKC-ζ signaling.

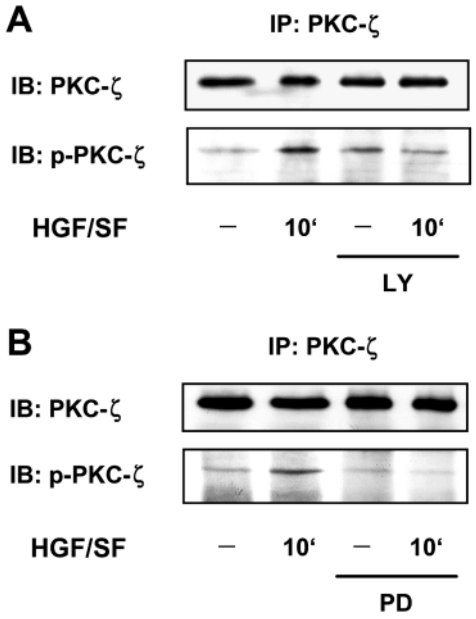

Fig. 10. Inhibition of PI 3-kinase (LY 294002) and MEK1 (PD 98059) prevents HGF/SF-induced phosphorylation of PKC-ζ. Western blot analyses of HaCaT cells that were left untreated (media change) or were exposed to HGF/SF (for 10 minutes at 100 ng/ml) after preincubation with (A) PI 3-kinase inhibitor LY 294002 (LY; at 10 μM for 60 minutes), with (B) MEK1 inhibitor PD 98059 (PD; at 50 μM for 60 minutes) or with solvent only (DMSO, 0.1%). Cellular extracts were immunoprecipitated (IP) by specific anti-PKC-ζ antibody (Santa Cruz) prior to immunoblotting (IB). Immunoblotting with pan-PKC-ζ antibody (upper panel) and with phospho-PKC-ζ antibody (lower panel). Immunoblots displayed are representative of two that were performed revealing comparable results.

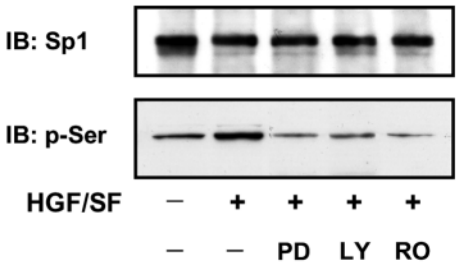

Fig. 11. HGF/SF-induced serine-phosphorylation of Sp1 is attenuated by PI 3-kinase, MEK1 and broad range PKC inhibition. Western blot analyses of HaCaT cells that were left untreated (media change, lane 1) or were stimulated by HGF/SF (for 10 minutes at 100 ng/ml) after preincubation with MEK1 inhibitor PD 98059 (PD; at 50 μM for 60 minutes), with PI 3-kinase inhibitor LY 294002 (LY; at 10 μM, for 60 minutes), with broad range PKC inhibitor RO 31-8220 (RO; at 20 μM for 60 minutes) or with solvent only (DMSO, 0.1%, lane 2). Cellular extracts were immunoprecipitated (IP) by specific anti-Sp1 antibody (Sigma) prior to immunoblotting (IB). Immunoblotting with a Sp1 antibody (upper panel) and with a phospho-serine monoclonal antibody (Biomol, clone 4A3; lower panel). Experiments were repeated twice with comparable results.

Discussion

VEGF/VPF has been identified as a key regulator of angiogenesis in malignancies and in a variety of non-neoplastic diseases (reviewed by Carmeliet and Jain, 2000; Folkman, 1995). Besides its significance as a tumor angiogenesis factor of general importance, convincing evidence established VEGF/VPF as a major driving force in multiple skin angiogenic responses (reviewed by Detmar, 2000). VEGF/VPF expression by epithelial derived tumor cells as well as epidermal keratinocytes is subject to regulation by a variety of distinct stimuli (reviewed by Dvorak et al., 1995; Ferrara, 1999). Accumulating data demonstrate that several angiogenesis factors themselves are potent inducers of VEGF/VPF gene expression, suggesting that their angiogenic properties may be mediated in part or amplified by inducing VEGF/VPF synthesis. Among the latter factors, HGF/SF has been demonstrated to strongly upregulate VEGF/VPF in different cell types (Dong et al., 2001; Van Belle et al., 1998). HGF/SF is a known angiogenesis factor discovered primarily through its direct effects on endothelial cell motility and capillary tube formation in vitro and its ability to induce angiogenesis in vivo (reviewed by Rosen and Goldberg, 1997). As a key paracrine mediator of mesenchymal epithelial interactions (reviewed by Birchmeier and Birchmeier, 1994; Rosen et al., 1994), its ability to induce VEGF/VPF expression in different cell types (Clifford et al., 1998; Moriyama et al., 1998) and to potentiate its angiogenic effect via paracrine upregulation of VEGF/VPF in vivo (Van Belle et al., 1998) strongly supports a model of an indirect amplification loop of angiogenesis. We therefore intended to elucidate the molecular mechanisms employed to mediate HGF/SF-induced VEGF/VPF gene expression.

Here, we provide evidence that HGF/SF-mediated VEGF/VPF gene transcription and subsequent protein expression may be conveyed via increased Sp1 phosphorylation as a way to enhance Sp1 transactivating activity. Previously, we showed that HGF/SF-induced VEGF/VPF expression by keratinocytes and epithelial-derived tumor cells is primarily dependent on transcriptional activation, localizing the HGF/SF-responsive region to a Sp1-site-containing region between bp –88 and –65 of the VEGF/VPF gene (Gille et al., 1998). This region is critical for both basal and HGF/SF-induced transcriptional activity. The Sp family members Sp1 and Sp3 bind to this element constitutively (Fig. 2); however the VEGF/VPF promoter is transactivated by HGF/SF in the absence of induced binding activity. Though less frequently reported, regulated Sp1-site-dependent transcription may be mediated also in the absence of changes in factor binding to different genes (Alliston et al., 1997; Black et al., 1999; Borroni et al., 1997). As our supershift EMSAs indicate that bound complexes comprise either Sp1 or Sp3 protein (Fig. 2), variations in DNA binding and/or association of additional transcription factors with Sp proteins may represent unlikely mechanisms of HGF/SF-induced VEGF/VPF transcription.

Sp1 and Sp3 are ubiquitously expressed transcription factors that recognize GC-rich sequences (Azizkhan et al., 1993) present in regulatory sequences of numerous housekeeping genes and genes involved in growth regulation and cancer (reviewed by Black et al., 2001; Philipsen and Suske, 1999). Whereas Sp1 has mostly been shown to act in a positive manner, Sp3 may function either as repressor or activator of Sp1-mediated activation, depending on both the promoter-

dependent and cellular context. Our co-expression experiments with Sp1 and Sp3 in Sp-deficient Schneider cells indicate that both factors are equally capable of activating the VEGF/VPF promoter through the HGF/SF response region (Fig. 3). The effects may be slightly different in pancreatic carcinoma cells, in which co-expression of Sp3 was recently reported to induce VEGF/VPF transactivation to a lesser extent than did Sp1 expression (Shi et al., 2001). The differences in our observation may be related to the different length of the utilized VEGF/VPF-promoter-based reporter gene construct or may be due to cell-dependent basal expression levels of the respective Sp factors. In addition, our studies reveal that Sp3 lacks repressive effects on Sp1-induced VEGF/VPF transcription. Hence, HGF/SF-induced Sp1 site-dependent VEGF/VPF transcription in HaCaT keratinocytes may not be conferred by relative changes of Sp1 and/or Sp3 DNA binding or by differing transactivating properties of the respective Sp family members on VEGF/VPF transactivation.

As a result, we favored the hypothesis that HGF/SF increased transactivation activity of constitutively expressed Sp family members as a molecular mechanism to induce VEGF/VPF gene transcription. Previously, it has been demonstrated that Sp1 is subject to several post-transcriptional modifications including glycosylation and phosphorylation (reviewed by Black et al., 2001; Philipsen and Suske, 1999). Sufficient evidence suggests that Sp1 phosphorylation may represent a means for regulating transcriptional initiation. In the current study, Sp1 immunoprecipitates were assayed by western blotting for the presence of serine- and threonine-phosphorylated proteins, revealing increased intracellular amounts of serine-phosphorylated Sp1 in response to HGF/SF (Fig. 4A). Therefore, of the potential parameters tested, neither alterations in expression and DNA-binding activity nor distinct transactivating properties of the respective Sp family members accounted for HGF/SF-induced Sp1 site-dependent VEGF/VPF transcription; only changes in Sp1 phosphorylation could account for it. Alhough the evidence presented is largely indirect, along with our supplementary findings as well as previously established mechanisms of Sp1-site-dependent transcription, an increase in Sp1's transactivation potential owing to changes in its phosphorylation state may provide a rational and likely conclusion by which HGF/SF conveys induced VEGF/VPF transcription.

Whereas Sp1 is ubiquitously expressed, activation and recruitment of distinct transduction pathways and signaling molecules may serve as mechanisms to promote transactivation of selected responsive genes. Sp1 is phosphorylated by a number of cellular kinases, including DNA-dependent protein kinase, protein kinase A (PKA) and different members of the PKC family (reviewed by Black et al., 2001). Among the PKC isoforms, atypical PKC-ζ has been implicated in Sp1-dependent VEGF/VPF gene expression (Pal et al., 1998; Pal et al., 2001). It has been indicated that Sp1 can bind to PKC-ζ and may act as a direct substrate for PKC-ζ. In addition, overexpression of PKC-ζ induced VEGF/VPF transcription in a Sp1-site-dependent fashion, whereas co-transfection of a dominant-negative PKC-ζ mutant repressed constitutive VEGF/VPF promoter activity concentration dependently. The importance of PKC-ζ was substantiated in a recent study on stretch-induced VEGF/VPF mRNA expression (Suzuma et al., 2002). Overexpression of kinase-deficient PKC-ζ by adenoviral transfer almost entirely blocked stretched-induced VEGF/VPF mRNA levels by bovine retinal pericytes and endothelial cells. However, expression of wild-type PKC-ζ did not induce VEGF/VPF transcript levels in these cell types. To determine the putative role of PKC-ζ in HGF/SF-induced VEGF/VPF expression, we studied the impact of antisense oligonucleotide transfection as well as wild-type and mutant PKC-ζ overexpression (Fig. 6B,C). These experiments suggest that PKC-ζ is critically involved in HGF/SF-induced VEGF/VPF expression by HaCaT keratinocytes. Inhibition of PKC-ζ significantly blocks induced VEGF/VPF transcriptional activity, whereas antisense oligonucleotides directed against conventional PKC-α fails to exert inhibitory effects in our transfection experiments. The contribution of PKC-ζ is underscored by findings showing prompt phosphorylation of PKC-ζ in response to HGF/SF and inhibition of induced VEGF/VPF expression by overexpression of mutant PKC-ζ (Fig. 6A). To further strengthen the significance of Sp1 phosphorylation in HGF/SF-induced VEGF/VPF gene expression, we determined the effect of broad range PKC inhibition by compound RO 31-8220 on HGF/SF-induced Sp1 phosphorylation. In agreement with our hypothesis, induced phosphorylation of Sp1 is prevented by broad range PKC inhibition (Fig. 11). As PKC-ζ has been previously shown to interact with and to phosphorylate Sp1 (Pal et al., 1998), PKC-ζ may be engaged as an essential downstream target of HGF/SF signaling in HaCaT cells.

To identify critical upstream signaling molecules of HGF/SF-induced VEGF/VPF promoter activation, the effects of specific pharmacological inhibitors and overexpression of kinase-deficient signaling proteins were studied. HGF/SF have been demonstrated to mediate its biological activities through binding to the Met receptor, activating a number of intracellular pathways (reviewed by Comoglio and Boccaccio, 2001; Stuart et al., 2000). Also, basal and induced VEGF/VPF gene expression has been shown to require distinct signaling modules, depending on the stimulus applied as well as on the cellular context chosen. MEK1/2, PI 3-kinase and different PKC isoforms belong to the major signaling molecules implicated in both HGF/SF-mediated responses and regulated VEGF/VPF expression. The PKC family currently consists of 12 isozymes, which are grouped in different functional classes, the conventional (cPKC-α, -β_I, -β_{II}, -ε), novel (nPKC-δ, -ε, -η, -θ), atypical (aPKC-ζ, -τ) and recently described PKC-μ and -ν on the basis of the structural differences that foster different requirements for activation by diacylglycerol and calcium (Dempsey et al., 2000; Newton, 1997). Earlier, HGF/SF treatment was reported to activate and translocate PKC-α, -ε and -γ in neocortical cells (Machide et al., 1998) and PKC-α, -β_{II} and -δ in a lung adenocarcinoma cell line (Awasthi and King, 2000). In addition, chemical inhibition of conventional/novel PKC isozymes was previously found to abrogate induced VEGF/VPF expression in different cell types (Hossain et al., 2000; Kim et al., 2000). In our studies, HGF/SF was shown to increase phosphorylation of novel PKC-δ in immortalized HaCaT keratinocytes, which was inhibited by preincubation with broad-range and isotype-specific conventional/novel PKC inhibitors (Fig. 5A). Though different PKC isozymes may be activated by HGF/SF, induced VEGF/VPF transcription and protein expression were not

affected by chemical PKC inhibition at concentrations that effectively blocked PKC phosphorylation (Fig. 5B,C). Whereas HGF/SF-mediated activation of different functional PKC isozymes has been associated with enhanced cell migration and growth (Cai et al., 2000; Chandrasekher et al., 2001) and has been linked to increased invasive potential of cancer cells (Kermorgant et al., 2001), conventional/novel PKC seems not to contribute to angiogenesis via HGF/SF-induced VEGF/VPF expression in keratinocyte-derived HaCaT cells.

The key signaling proteins MEK1/2 and PI 3-kinase have been clearly linked to both HGF/SF-mediated responses and induced VEGF/VPF expression. We assessed whether these kinases are activated by HGF/SF in HaCaT keratinocytes and whether their explicit inhibition affected HGF/SF-induced VEGF/VPF expression. Our data demonstrate that ERK1/2 and Akt as the respective downstream targets of MEK1/2 and PI 3-kinase are phosphorylated in response to HGF/SF (Figs 7 and 8). Specific pharmacological inhibition of MEK1/2 and PI 3-kinase blocked activation of their substrates and also abrogated HGF/SF-induced VEGF/VPF promoter activity and protein expression, indicating a critical contribution of both signaling modules. These findings are in line with a most recent report on HGF/SF-induced VEGF/VPF mRNA and protein expression in head and neck squamous carcinoma (Dong et al., 2001), indicating that the HGF/SF induction pathway may be not be restricted to keratinocytes only but may be functional in different epithelial cells. As chemical inhibition of either PI 3-kinase or MEK1/2 resulted in abrogation of induced VEGF/VPF expression, the question arose as to whether there is crosstalk between the two signaling pathways or whether they are connected sequentially to induce VEGF/VPF expression. Previously, it has been reported that MEK1/2 inhibition can diminish HGF/SF-induced Akt phosphorylation, as a substrate of PI 3-kinase (Delehedde et al., 2001; Yu et al., 2001), or vice versa. PI 3-kinase inhibition may also block HGF/SF-induced ERK1/2 activation, as a substrate of MEK1/2 (Delehedde et al., 2001; Sipeki et al., 2000). In our studies, however, neither MEK1/2 inhibition blocked Akt phosphorylation nor PI 3-kinase inhibition reduced ERK1/2 activation (Fig. 9), whereas phosphorylation of the corresponding substrates was abrogated. Mechanistically, PI 3-kinase and MEK1/2 thus appear to mediate the HGF/SF signal partly in parallel in HaCaT keratinocytes without crosstalk, as HGF/SF-induced activation of immediate PI 3-kinase and MEK1/2 downstream targets is mutually independent. Nonetheless, both signaling modules appear to jointly block shared downstream signaling protein(s), since pharmacological inhibition of each pathway almost completely blocked HGF/SF-mediated VEGF/VPF transcription and protein expression. A comparable signaling scenario seems to be in place to mediate anti-apoptotic HGF/SF effects in different cell lines (Xiao et al., 2001). PKC-ζ may serve as a downstream effector of MEK1 and PI 3-kinase upon HGF/SF-stimulation, since phosphorylation of respective downstream targets occurs despite the presence of overexpressed kinase-deficient PKC-ζ (Fig. 9). This assumption is also supported by experimental evidence demonstrating that HGF/SF-induced activation of PKC-ζ is efficiently blocked by both PI 3-kinase and MEK1/2 inhibition (Fig. 10A,B). These results suggest that signaling pathways employed to direct HGF/SF biological effects are both stimulus and cell context dependent.

Pertinent to this conclusion, regulated VEGF/VPF gene expression has also been shown to be conferred via activation of distinct signaling molecules, depending on the cellular context and on the inducing stimulus. Since the first description of the significance of the Sp1 site cluster in close proximity to the transcription start (Gille et al., 1997; Ryuto et al., 1996), this region has been implicated in the regulation of VEGF/VPF gene transcription in response to several important stimuli (Finkenzeller et al., 1997; Gille et al., 1998; Tanaka et al., 2000). Moreover, transrepression of VEGF/VPF promoter activity by von Hippel-Lindau and p53 tumor suppressor gene product was shown to be mediated via interaction with this GC-rich region (Mukhopadhyay et al., 1997; Zhang et al., 2000). Alhough many different stimuli for VEGF/VPF induction have been identified in the past, studies on comprehensive evaluation of involved upstream signaling molecules have only recently been reported. Activation of VEGF/VPF promoter activity by MEK1 overexpression was shown to depend on the GC-rich –88/-66 bp promoter element in lung fibroblasts. By contrast, overexpression of c-Jun N-terminal kinase (JNK) or p38 MAPK as well as their activating kinases had no effect on VEGF/VPF promoter activity in this experimental set-up (Milanini et al., 1998). Additionally, overexpression of Ras has been shown to mediate Sp1-dependent transcriptional activation of VEGF/VPF, involving PI 3-kinase and PKC-ζ as important intermediary signaling molecules (Pal et al., 2001). The contribution of MEK1/2 as a potential target protein of Ras signaling was not investigated in these studies, however. PI 3-kinase may also be involved in hypoxia-induced VEGF/VPF gene expression, as PI 3-kinase inhibition partially blocked VEGF/VPF in epithelial cells (Blancher et al., 2001). In Ha-Ras-transformed fibroblasts, inhibition of PI 3-kinase even completely prevented hypoxia-mediated VEGF/VPF expression (Mazure et al., 1997). Notably, the hypoxia-response element is located far more upstream in the VEGF/VPF promoter at bp –985/-939, requiring the interaction with hypoxia-inducible factor-1 (Forsythe et al., 1996; Liu et al., 1995). VEGF/VPF activation via different response elements may thus be channeled through similar signaling pathways. The molecular mechanisms involved in induced VEGF/VPF expression by a given stimulus are therefore not predictable, as they may involve PI 3-kinase/PKC-ζ but not MEK1/2 (e.g. stretch) (Suzuma et al., 2002) or they may depend on p38 MAPK but not on MEK1/2 and PI 3-kinase (e.g. heregulin-β1) (Xiong et al., 2001). Consequently, the cellular context and the induction pathways have to be considered to define appropriate targets in order to intersect with the regulated VEGF/VPF expression as a denominator of angiogenesis.

Together, we demonstrate that both MEK1/2 and PI 3-kinase contribute to HGF/SF-induced VEGF/VPF promoter activation and protein expression by keratinocytes. We herein provide thorough evidence that HGF/SF increases the intracellular amount of serine-phosphorylated Sp1, most likely by engaging PKC-ζ as a downstream intermediate signaling protein. Our findings characterize a novel potential mechanism of paracrine HGF/SF-induced upregulation of VEGF/VPF expression that permits a more detailed understanding of a pathway implicated in indirect amplification of angiogenesis.

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