The Hepatocyte Growth Factor Receptor Kinase-mediated Phosphorylation of Lipocortin-1 Transduces the Proliferating Signal of the Hepatocyte Growth Factor*

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George G. Skouteris‡ and Claus H. Schröder

From the Division of Virus-Host Interactions, Research Program of Applied Tumor Virology, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

The hepatocyte growth factor/scatter factor (HGF/SF) receptor is a tyrosine kinase encoded by the c-met oncogene (1, 2). The HGF/SF receptor tyrosine kinase (MET) is heterodimeric composed of an extracellular α and a transmembrane β disulfide-linked subunits. The latter subunit is composed of a cytoplasmic portion containing the tyrosine kinase domain and an extracellular part containing the binding site for extracellular ligands (3). Recently, it has been reported that a MET homologue, RON, encodes a tyrosine kinase activated by the macrophage stimulatory protein (MSP) (4). Binding of the HGF/SF to its receptor (MET), triggers autophosphorylation of the p145MET, which is a common step preceding the various biochemical responses elicited by HGF/SF, such as mitogenesis, motogenesis, and matrix invasion (5–8). The HGF/SF receptor tyrosine kinase activity is positively regulated by autophosphorylation presumably on Tyr1235, whereas it is negatively regulated by phosphorylation by activated protein kinase C and by increased calcium release from intracellular stores (9). Upon autophosphorylation p190MET was reported to associate in vitro with various transducers containing SH2 domains and among them with the 85-kDa subunit of the phosphoinositide 3-kinase, the phospholipase Cγ, p59Fyn, Src, and others (10–12).

Prostaglandins have been implicated in various cellular functions, such as the regulation of mitogenesis in primary hepatocytes and in A549 cells, a lung carcinoma cell line, which is known to overexpress the HGF/SF receptor (5000 receptors/cell) (13–15). The rate-limiting step for prostaglandin production is believed to be the liberation of arachidonic acid by the membrane phospholipid through action of phospholipase A2 (cPLA2) (16). Arachidonic acid can also be produced by activation of phospholipase C and diacylglycerol lipase followed by its activity on diacylglycerols (17).

Lipocortin-1 (LC-1) is an approximately 38-kDa protein that has been proposed to be a putative mediator of the anti-inflammatory actions of glucocorticoids (18). The LC-1 contains a core that is responsible for calcium and phospholipid binding. This core is part of the N terminus, which also contains tyrosine and serine residues as potential phosphorylation sites for protein kinase C and the EGF-receptor kinase (19). By peptide mapping it has also been shown that the unique tyrosine residue that is phosphorylated by the EGF receptor kinase is actually located in the N terminus (Tyr21) (20). Phosphorylation of lipocortin-1 results in the release of cPLA2 activity, rendering this enzyme activated and therefore implicated in the regulation of prostaglandin-associated processes (21). These structural and functional characteristics of lipocortin-1 implicate this protein in the intracellular transduction of mitogenic signals.

In this study we investigated whether the stimulation of the A549 cells with HGF/SF would involve changes in the synthesis and the phosphorylation state of LC-1 and thereby could modulate cellular proliferation.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were purchased from Sigma, and radioactive compounds were from Amersham International, United Kingdom. HGF/SF was a kind gift of Dr. R. Schwall, Genentech, CA, and mouse met (pMMET) was a kind gift of Dr. G. F. Vande Wounde, National Institutes of Health.

Methods

Cell Culture, Proliferation, and PGE2 Assay—A549 cells were obtained from Dr. I. Freshney (Glasgow, United Kingdom) cultured in
Dulbecco’s modified Eagle’s medium:F-12 1:1 (Life Technologies, Inc.) containing 10% fetal calf serum (FCS). Cells were maintained in serum-supplemented medium containing 95%:5% air-CO₂ humidified atmosphere. Viability was assessed routinely using trypan blue exclusion test, and cell proliferation was estimated by treating cells with 0.5% trypsin, 0.02% EDTA in phosphate-buffered saline and counting using a Neubauer hemocytometer or a Coulter counter. For DNA synthesis experiments, [methyl-³H]thymidine (2 μCi/dish, specific activity > 75 Ci/mmol) was added to the cultures for 2 h. DNA and radioactivity were estimated as described previously (14). Medium from HGF/SF-treated cultures with or without additional additions was collected, stored frozen (–70°C), and the PGE₂ content was measured using a commercially available radioimmunoassay kit (Amerham International). Where appropriate, immunoblots were stripped in 62.5 mM Tris-HCl pH 6.7, containing 2% SDS and 100 mM β-mercaptoethanol, for 30 min at 50°C.

RESULTS
HGF/SF Induces Proliferation of A549 Cells and Stimulates Production of PGE₂—HGF/SF was added in 2-day serum-starved A549 cells at concentrations between 10–500 ng/ml. Cell proliferation was monitored by counting the cell number at various points after HGF/SF addition. The cell number of A549 serum-starved cells treated without HGF/SF or FCS throughout the culture time was detected always below that found at the 3rd day under HGF/SF treatment (not shown). HGF/SF-induced proliferation of A549 cells began 48 h after its addition. This factor was effective above 10 ng/ml; however, HGF/SF was used at 50 ng/ml throughout the cell proliferation experiments. On the 10th day in culture, the cell number was 4-fold the control number (Fig. 1A). HGF/SF stimulated the proliferation of A549 cells in a manner similar to that of 10% FCS. However, between 1 and 6 days in culture, the magnitude of HGF/SF stimulation was higher than that induced by FCS. The HGF/SF was tested at concentrations between 10 and 500 ng/ml. Cell proliferation of the A549 cells reached a plateau at HGF/SF concentrations between 50 and 100 ng/ml. This was tested during the 5th and 6th day in culture (Fig. 1B).

It has been shown that in A549 cells the release of PGE₂ plays a role in their growth regulation (27). We have tested the capacity of HGF/SF treatment to induce production of PGE₂ (Fig. 1C). The amount of PGE₂ released into the medium on the 3rd day in culture was 6.4 ng/ml, and on the 6th, 9th, and the 12th day the amount of PGE₂ released into the medium was 7.1, 9.3, and 24.8 ng/ml, respectively. Control values from starved cells in the absence of growth factors or FCS were below 1 ng/ml (Fig. 1C). To explore whether the HGF/SF activity on cell proliferation was directly correlated with the release of PGE₂, we included in the medium an anti-PGE₂ antibody (10 μg/ml) 2 h before the addition of HGF/SF. The proliferation of A549 cells was markedly decreased, thus showing that the released prostaglandin caused a receptor-mediated response leading to A549 proliferation (Fig. 1D). The presence of an antibody against MET (10 μg/ml) in the medium, also added 2 h before the addition of HGF/SF, inhibited the proliferation of the A549 cells at an extent similar to that observed in the presence of the PGE₂ antibody. However, inclusion of a well characterized anti-MYC monoclonal antibody (CT14.3T3, ATCC) in the A549 cultures did not affect the cell number nor the viability of the cells in the presence or not of HGF/SF (Fig. 1D). It should also be pointed out that p67/69 MYC detected by immunoblotting was poorly expressed in A549 cells regardless of whether HGF/SF was present or not (not shown).

Pulse labeling with [³H]Tdr for 2 h confirmed the results on cell proliferation revealed by counting the cell number. To further test the data on the correlation between HGF/SF stimulation and PGE₂ release, indomethacin a cyclooxygenase inhibitor was added 1 day after the addition of HGF/SF and cell proliferation was estimated by [³H]Tdr incorporation (Fig. 1D). Indomethacin was effective at concentrations between 0.1 and 100 μM. Specifically, cell proliferation was inhibited by 80.3% at the 4th day using indomethacin at 10 μM and beyond 90% at the 5th and the 6th day in culture. Inhibition was partially reversed by co-addition of PGE₂ (100 μM) and indomethacin (10 μM) (42% inhibition the 6th day, not shown). Addition of verapamil at 50 μM the 3rd day in culture in the presence of HGF/SF resulted in inhibition of the A549 prolif-
peration (Fig. 1D). Verapamil was effective at 5 nM; when PGE2 (100 pM) was coated with verapamil (50 nM) in A549 cells treated with HGF/SF, it caused a partial reverse of the observed inhibition (52% of the control value the 5th day estimated as [3H]Tdr incorporation, not shown). Neither verapamil nor indomethacin affected cell viability, as shown by routine trypan blue exclusion testing.

Activation of the p145\textsuperscript{MET} Induces Tyrosine Phosphorylation of Lipocortin-1—

Addition of HGF/SF to serum-starved A549 cultures induced phosphorylation on tyrosine residues (detected using an anti-phosphotyrosine antibody) of cellular species with apparent molecular masses of 145, 85, 69, and 38 kDa (Fig. 2A). Stripping of the immunoblots and re-probing with an anti-MET antibody (C28) raised against the extracellular domain of the MET receptor has revealed the identity of the 145-kDa phosphorylated species with the \textbeta-subunit of the HGF/SF receptor (Fig. 2B). One-half of the lysate was immunoblotted (Fig. 2A), and the other half was immunoprecipitated with an anti-lipocortin-1 monoclonal antibody, immunoblotted, and probed with the PY-20 antibody (anti-phosphotyrosine monoclonal antibody, Dianova) (Fig. 2C). The 38-kDa tyrosine-phosphorylated protein was shown to be identical to lipocortin-1, and its synthesis was slightly induced, shortly after addition of HGF/SF (30 min) (Fig. 2D). It should also be noted that although the relative amounts of lipocortin-1 were practically unaltered up to 4 h after addition of HGF/SF, phosphorylation of LC-1 reached maximal levels, after only 1 h (Fig. 2C). Preincubation of the serum-starved A549 cells with the anti-MET antibody for 2 h prior to the addition of HGF/SF, or treatment of the cells with genistein (an inhibitor of tyrosine phosphorylation), abolished phosphorylation of all protein species, thus indicating that the observed tyrosine phosphorylation was primarily due to the activated HGF/SF receptor kinase.

Effects of HGF/SF on the Synthesis, Phosphorylation, and Translocation of Lipocortin-1—

Addition of HGF/SF to serum-starved A549 cells induced phosphorylation of the p145\textsuperscript{MET} (Fig. 3A). Probing of immunoblotted A549 proteins with the PY20 monoclonal antibody revealed that p145\textsuperscript{MET} phosphorylation lasted for up to 120 min after addition of the ligand (Fig. 3A). We did not observe differences in the degree of phosphorylation of p145\textsuperscript{MET} in cultures treated with HGF/SF and those treated with HGF/SF together with dexamethasone (1 \textmuM). In contrast to other systems (28), in A549 cells this glucocorticoid did not alter the phosphorylation state of the p145\textsuperscript{MET} (Fig. 3A). After 4 h, p145\textsuperscript{MET} phosphorylation was returned to control levels. Addition of HGF/SF in serum-starved A549 cells did not induce changes in total cell lipocortin-1 levels for up to 24 h (Fig. 3B). Dexamethasone, which was shown to induce the synthesis of lipocortin-1 in A549 cells (16), when present to-
stripping with the anti-lipocortin-1 antibody (D) and re-probing with anti-MET rabbit polyclonal antibody (C28) is shown in B. One-half of the immunoblotted lysate shown in A was immunoprecipitated with anti-lipocortin-1 antibody, immunoblotted, and probed with PY-20 (C) and after stripping with the anti-lipocortin-1 antibody (D). At the right side of the immunoblots, molecular masses and names of the reacting species are shown. The data are representative of two independent experiments.

P-Tyr, phosphotyrosine.

gather with HGF/SF, did not seem to modify significantly lipocortin-1 levels. However, 48–72 h after co-addition of HGF/SF and dexamethasone, the level of lipocortin-1 was significantly increased in comparison with the levels observed for up to 24 h. In the presence of HGF/SF, phosphorylation of lipocortin-1 was shown to be significant for up to 24 h, and co-addition of dexamethasone did not alter the phosphorylation state of this protein (Fig. 3C). In serum-starved cells lipocortin-1 was located on the membrane fraction and translocated again to cytosol, 12 h after HGF/SF addition. Therefore, stimulation with HGF/SF induced an early translocation of lipocortin-1 to the membrane fraction, data indicating a role for this protein in HGF/SF signal transduction. The data presented in Figs. 2 and 3 clearly suggest that HGF/SF promotes via activation of the p145βMET tyrosine kinase activity, phosphorylation of lipocortin-1 on tyrosine.

The p145βMET Subunit Associates in Vivo with Lipocortin-1—Cell lysates from serum-starved A549 cells, stimulated with or without HGF/SF, were immunoprecipitated with anti-lipocortin-1 monoclonal antibody, and the eluted proteins were immunoblotted and probed with the anti-PY20 antibody (Fig. 4A). Most of the tyrosine-phosphorylated lipocortin-1 was detected in lysates from HGF/SF-treated cells (Fig. 4A, lane a). Dexamethasone did not affect the phosphorylation of immunoprecipitated protein species originating from HGF/SF-treated cells (Fig. 4A, lane b). Stripping of the immunoblot and re-probing with the anti-lipocortin-1 antibody showed that similar amounts of lipocortin-1 were eluted from the immunoprecipitates, and subsequent re-probing with the anti-MET antibody (C28) MET-related species was identified. These data clearly indicated that lipocortin-1 co-precipitates with p145βMET, and this in vivo association is independent of the phosphorylation state of the receptor’s β-subunit.

In an experiment run in parallel, subconfluent (80%) NIH3T3 and MRC-5 fibroblasts, as well as the stable NIH3T3 met transformants, were stimulated with 100 ng/mL of HGF/SF and immunoprecipitated with anti-lipocortin antibody (Fig. 4D). Probing of the immunoblotted species with anti-MET (C28), and anti-lipocortin-1 antibodies, revealed no MET species corresponding to the MET apparent molecular weight in the NIH3T3 and the MRC-5 immunoprecipitates (Fig. 4E).

The p145βMET Phosphorylates Endogenous Lipocortin-1 in Vitro—Membranes isolated from HGF/SF-stimulated or unstimulated A549 cells were used as the source of kinase activity for in vitro phosphorylation assays (Fig. 5A). Membranes isolated from serum-starved and unstimulated A549 cells failed to induce phosphorylation of any endogenous substrate in the presence of [γ-32P]ATP (Fig. 5A, lane a). The major endogenous substrate, which was phosphorylated by HGF/SF-stimulated membranes, was identified to be lipocortin-1. Phosphorylation of membrane-associated lipocortin-1 by the β-subunit of the receptor was complete when A549 cells were stimulated with HGF/SF for 10 min (Fig. 5A, lane b). Membranes isolated from serum-starved and unstimulated A549 cells failed to induce phosphorylation of any endogenous substrate in the presence of [γ-32P]ATP (Fig. 5A, lane a). In the presence of membranes from HGF/SF-stimulated A549 cells, phosphorylation of other endogenous substrates of apparent molecular masses of 12, 21, 48, and 85 kDa was also induced. Inclusion of Triton X-100 in the kinase reaction (to identify if intact membranes are required for phosphorylation) did not inhibit phosphorylation of either lipocortin-1 or of p 145βMET. Co-inclusion of Triton X-100 and Nonidet P-40 in the phosphorylation reaction resulted in a 72% inhibition of lipocortin-1 phosphorylation (as assessed by densitometric scanning) (Fig. 5A, lanes d and e). The residual phosphorylation of the 38-kDa lipocortin-1, in the presence of Triton X-100 alone, is attributed to partial solubilization of the membrane fraction which, however, was completed when Nonidet P-40 was included.

When exogenous calcium chloride was introduced in the reaction at three different concentrations (100 μM, 1 mM, and 10 mM), phosphorylation of both the p145βMET and of lipocortin-1 was altered, and phosphorylation of species migrating at 12, 21, 48, 68/69, and 85 kDa was markedly increased proportionally to the amount of exogenous calcium added (Fig. 5A, lanes g–i). Inclusion of calcium at increasing concentrations did not affect or slightly increased (at 10 mM) p145βMET phosphorylation. Lipocortin-1 phosphorylation was decreased compared with controls (Fig. 5A, lanes g–i and b and c), and this can be attributed to the activation of calcium-dependent protein kinase(s), which in turn can phosphorylate various endogenous substrates. In addition, activated calcium-dependent protein kinase(s) by the increased calcium influx have been reported to induce serine phosphorylation of p145βMET, thus down-regulating the tyrosine kinase activity of the receptor’s β-subunit (36).

In the presence of EGTA (2 mM), phosphorylation of all endogenous species in kinase reactions supplemented or not with exogenous calcium was abolished almost to completion, including that of lipocortin-1 (Fig. 5A, lane f). Co-addition of calcium chloride (1 mM) and EGTA (2 mM) in the kinase reaction partially restored the phosphorylation of lipocortin-1 (Fig. 5A, lane j). These results suggest that p145βMET tyrosine kinase has a calcium requirement for its activity. Presence of anti-MET antibody originating from two different sources (Ab2: C28 and Ab3:Genentech), or of anti-lipocortin-1 antibody, caused a significant decrease in the phosphorylation of both lipocortin-1 and of other protein species (Fig. 5A, lanes k–m). Immunoblotting of the kinase reaction gel with either anti-MET or after stripping with anti-lipocortin-1 antibodies
revealed that equal levels of MET-reacting species (p190, p145) and lipocortin-1 were included in the kinase reactions, except in those reactions where anti-MET or anti-lipocortin-1 antibodies were added (Fig. 5, B and C).

**Antisense Oligonucleotides to Lipocortin-1 Inhibit p145β-MET-induced Phosphorylation of Lipocortin and A549 Cell Proliferation—**The 21-mer antisense lipocortin-1 oligonucleotide (AsLip) or its scrambled version (ScrLip) were used at 50 or 150 nM to study their effects on the HGF/SF-induced phosphorylation of lipocortin-1 and on A549 cell proliferation (20, 22) (Figs. 6 and 7). Two-day serum-starved A549 cells were treated with either AsLip or ScrLip 24 h before the addition of HGF/SF (50 ng/ml for 30 min), in the presence or not of anti-lipocortin-1 antibody. Membranes were isolated, and kinase reactions were carried out in the presence of [γ-32P]ATP (Fig. 6A). Phosphorylation of lipocortin-1 was significantly reduced when in the kinase reactions the membranes used (tyrosine kinase source) were from A549 cells treated with AsLip at both concentrations used. However, in ScrLip-treated A549 cells, phosphorylation of lipocortin-1 was restored almost to control levels (Fig. 6A, lanes b and e, and Fig. 5, lane c). The decrease in lipocortin-1 phosphorylation was accompanied by a decrease in the levels of this protein detected in membranes isolated from AsLip-treated cells (AsLip 519% of the ScrLip-treated, as assessed by densitometric scanning) (Fig. 6B, lanes a and b). Co-addition of a lipocortin-1 antibody with the AsLip oligonucleotide and preincubation for 24 h before the addition of HGF/SF resulted in almost complete inhibition of lipocortin-1 phosphorylation, thus indicating the ability of the antibody to neutralize the membrane-associated or the extracellular lipocortin-1 (Fig. 6A, lanes f and g).

**met** species from stable HGF/SF-treated NIH3T3 met transformants were affinity-purified and then tested for their ability to possess functional tyrosine kinase activity. met species were immunoprecipitated and collected in Protein A-agarose. In vitro kinase reactions were then carried out with the immobi-
from HGF/SF-treated NIH3T3 precipitates. The MET-immunoprecipitated species, however, kinase reactions carried out phosphorylated the reacted species were collected on Protein A-agarose and immunoprecipitated with either anti-MET or anti-MYC antibodies. Membrane-stimulated HGF/SF receptor in the presence of \( ^{32}P \)ATP as described. Triton X-100 (1% in NP-40 (100 mM)) and Nonidet P-40 (100 mM CaCl₂, 50 mM EGTA, or 0.1% TX-100) were used at 0.5% each. EGTA was added at 2 mM, and each of the antibodies was used at 1 µg/assay. The phosphorylated species shown in A were immunoblotted and probed with anti-MET (C28) or anti-lipocortin-1 antibodies (following stripping). Reacting species were revealed by ECL, and molecular masses are shown in kilodaltons on the right side. The data presented are representative of at least three independent experiments.

antimetas, stimulated with HGF/SF (100 ng/ml, 30 min) were immunoprecipitated with either anti-MET or anti-MYC antibodies. The reacted species were collected on Protein A-agarose and phosphorylated in vitro. Treatment of parental NIH3T3 cells with HGF/SF did not induce phosphorylation of proteins in kinase reactions carried out in vitro in MET or MYC immunoprecipitates. The MET-immunoprecipitated species, however, from HGF/SF-treated NIH3T3 met transformants have shown actively phosphorylated p145\(^{MET}\), whereas in MYC immunoprecipitates phosphorylation of p145\(^{MET}\) was undetectable (Fig. 6D).

Serum-starved A549 cells were treated with the AsLip or the ScrLip oligonucleotides at 150 nM, 24 h before addition of HGF/SF (50 ng/ml). The cell number was determined at different time intervals, and these results are shown in Fig. 7. There was a time-dependent decrease in cell number in A549 cells treated with the AsLip, whereas ScrLip the oligonucleotide did not seem to affect cellular proliferation (Fig. 7A). Co-addition of an anti-lipocortin-1 monoclonal antibody, together with AsLip or ScrLip, significantly reduced the cell number determined at the 6th day (compared with controls treated with HGF/SF alone). Media from A549 cultures treated as in Fig. 7A, were measured for PGE\(_2\) content. AsLip-treated A549 cells were shown to release significantly lower amounts of PGE\(_2\) in the medium compared with control levels. Co-addition of AsLip and lipocortin-1 antibody caused marked decrease in PGE\(_2\) release by the A549 cells (Fig. 7, lower panel). From the data shown in Figs. 6 and 7, it can be suggested that the reduction in lipocortin-1 phosphorylation is correlated to the reduced amount of the protein, due to its synthesis inhibition induced by the antisense oligonucleotide and to its availability due to antibody neutralization.

**FIG. 6.** Antisense-lipocortin-1 oligonucleotides modulate p145\(^{MET}\)-induced lipocortin-1 phosphorylation and purified HGF/SF-receptor tyrosine kinase phosphorylates in vitro recombinant lipocortin-1. Serum-starved A549 cells were treated for 24 h before addition of HGF/SF, with antisense (AsLip) or scrambled (ScrLip) oligonucleotides (21-mer, 4–24 base pairs) at the indicated concentrations. Membranes, isolated as described, were used for in vitro kinase reactions (A). The phosphorylated species were immunoblotted and probed with anti-lipocortin-1 antibody (B). C, affinity-purified HGF/SF receptor from stable NIH3T3 met transformants was used as the kinase source and following immunoprecipitation with anti-MET antibody (C28) was immobilized in protein A-agarose. The immobilized MET species were incubated for 1 h at 4 °C with the indicated amounts of purified recombinant mouse lipocortin-1. The complexes, after washing twice with lysis buffer and once with kinase buffer, were phosphorylated in vitro in the presence of \( ^{32}P \)ATP as described. D, parental NIH3T3 cells or NIH3T3 met transformants were immunoprecipitated with anti-MET (C28) or anti-MYC (CT14.GT3) antibody, the reacted species collected on protein A-agarose and phosphorylated in vitro. Molecular masses shown are expressed in kilodaltons.

**FIG. 5.** Phosphorylation of endogenous lipocortin-1 by the membrane-associated p145\(^{MET}\) tyrosine kinase activity. A, membranes from serum-starved A549 cells were prepared after a 30-min stimulation with HGF/SF (50 ng/ml) and used as a kinase source in in vitro kinase reactions in the presence of \( ^{32}P \)ATP as described. Triton X-100 (1%) and Nonidet P-40 (100 mM) were used at 0.5% each. EGTA was added at 2 mM, and each of the antibodies was used at 1 µg/assay. The phosphorylated species shown in A were immunoblotted and probed with anti-MET (C28) or anti-lipocortin-1 antibodies (following stripping). Reacting species were revealed by ECL, and molecular masses are shown in kilodaltons on the right side. The data presented are representative of at least three independent experiments.
transmembrane tyrosine kinase (MET) (1-3). HGF/SF promotes the involvement of multiple signaling pathways in the transduction of its signals, resulting in multilateral biological responses such as cell proliferation and scattering. It has been reported that EGF, transforming growth factor-α, and recently the HGF/SF-induced hepatocyte proliferation, are inhibited by a cyclooxygenase inhibitor, indomethacin (13, 14, 31), and furthermore that prostaglandins E₂ and F₂α play a significant role as inducers of hepatocyte proliferation acting in a direct or indirect manner (14, 32). Induction of A549 proliferation by a cyclooxygenase inhibitor, indomethacin (13, 14, 31), and furan derivatives such as 15-deoxy-a,β-dihydroxy-prostaglandin J₂ (15), which is thought to be a key enzyme in the metabolism of arachidonic acid (17). The role of lipocortins as mediators of prostaglandin metabolism has been reported that EGF stimulation was able to induce complete phosphorylation of cPLA₂ within 10 min, by a direct and not indirect manner (via lipocortin-1 phosphorylation) (17), thus ruling out involvement of lipocortin-1 phosphorylation in releasing cPLA₂ activity. Direct phosphorylation (on Ser⁵⁶⁹), and activation of cPLA₂ was shown to be effected by a mitogen-activated protein kinase (MAP kinase), which was upstream-activated by a protein kinase C-dependent or independent pathway (34). Activation of MAP kinases(s) by HGF/SF and by basic fibroblast growth factor was recently reported in primary hepatocytes and in endothelial cells, respectively, and to the activation of MAP kinase in turn activating cPLA₂ and subsequent release of arachidonic acid (31, 35). Taken together, we may suggest that both mechanisms releasing cPLA₂ activity (lipocortin-1 phosphorylation/MAP kinase activation of cPLA₂) may co-operate, depending upon the cellular system in response to the same ligand (HGF/SF). Preincubation of A549 cells with verapamil (50 nM) for 1 h, and subsequent stimulation with HGF/SF, resulted in decreased tyrosine autophosphorylation of p145MET, thus implying that replenishment of cellular calcium down-regulated the β-subunit tyrosine kinase activity. Inclusion of exogenous calcium chloride in the in vitro kinase reactions induced phosphorylation of several other than lipocortin-1 protein species, some of them found phosphorylated on tyrosine (not shown). Phosphorylation of these species may in part be attributed to downstream activation of calmodulin-dependent protein kinase(s), which, however, have been reported to decrease the receptor’s tyrosine kinase activity via phosphorylation of p145MET on Ser⁵⁶⁹ (36).

We have been unable to detect dramatic changes in lipocortin-1 levels in HGF/SF-stimulated A549 cells in the presence of dexamethasone, at early times after the addition of the effectors (HGF/SF + dexamethasone). In addition, dexamethasone failed to inhibit HGF/SF-induced lipocortin-1 phosphorylation throughout, although in other models, glucocorticoids were reported to inhibit kinases and to activate protein phosphatase 2A or 1 (28). HGF/SF-induced translocation of lipocortin-1 to the membrane fraction of A549 cells lasted for up to 6 h after ligand stimulation. Translocation of lipocortin-1 to the membrane fraction by dexamethasone has been reported for U-937 cells, and this event is believed to precede the extracellular release of this protein, in order to exert its anti-inflammatory activities (37). However, the data on the extracellular functions of this protein (lipocortin-1) still remain unclear. Despite the experimental evidence on lipocortin-1 translocation to the membrane fraction induced in the presence of HGF/SF, we believe that both cytosolic and membrane-associated lipocortin-1 are targets for phosphorylation by the β-subunit of the c-met oncogene, which so far is the only identified receptor for HGF/SF. This system (HGF/SF and its receptor) is operating in many animal tissues and among them in the liver and in the lung. In regenerating liver after partial hepatectomy, a model where HGF/SF and MET have a clear involvement in tissue remodeling, the time of the onset of DNA synthesis and the time of increased synthesis of lipocortins were reported to be identical (33).

Phosphorylation of lipocortin-1 releases cPLA₂ activity and HGF/SF in our study was shown to induce a rapid increase (within 10 min) in lipocortin-1 phosphorylation reaching maximal levels 1 h post-stimulation with HGF/SF. This is the first evidence for rapidly phosphorylated lipocortin-1 by growth factor receptors endowed with tyrosine kinase activity. Previous reports have shown that EGF stimulation was able to induce complete phosphorylation of cPLA₂ within 10 min, by a direct and not indirect manner (via lipocortin-1 phosphorylation) (17), thus ruling out involvement of lipocortin-1 phosphorylation in releasing cPLA₂ activity. Direct phosphorylation (on Ser⁵⁶⁹), and activation of cPLA₂ was shown to be effected by a mitogen-activated protein kinase (MAP kinase), which was upstream-activated by a protein kinase C-dependent or independent pathway (34). Activation of MAP kinases(s) by HGF/SF and by basic fibroblast growth factor was recently reported in primary hepatocytes and in endothelial cells, respectively, and to the activation of MAP kinase in turn activating cPLA₂ and subsequent release of arachidonic acid (31, 35). Taken together, we may suggest that both mechanisms releasing cPLA₂ activity (lipocortin-1 phosphorylation/MAP kinase activation of cPLA₂) may co-operate, depending upon the cellular system in response to the same ligand (HGF/SF). Preincubation of A549 cells with verapamil (50 nM) for 1 h, and subsequent stimulation with HGF/SF, resulted in decreased tyrosine autophosphorylation of p145MET, thus implying that replenishment of cellular calcium down-regulated the β-subunit tyrosine kinase activity. Inclusion of exogenous calcium chloride in the in vitro kinase reactions induced phosphorylation of several other than lipocortin-1 protein species, some of them found phosphorylated on tyrosine (not shown). Phosphorylation of these species may in part be attributed to downstream activation of calcium-dependent protein kinase(s), which, however, have been reported to decrease the receptor’s tyrosine kinase activity via phosphorylation of p145MET on Ser⁵⁶⁹ (36).
HGF/SF receptor.

The in vitro association of lipocortin-1 with the HGF/SF receptor, in a fashion independent of the phosphorylating state of the receptor, is observed for first time for p145βMET-associated intracellular effectors (transducers). Distinct phosphoryrse residues within the HGF/SF receptor domain(s) constitute binding sites for cytoplasmic transducers via the receptor's or the transducer's –SH2 domains (24). Such transducers have been identified to associate only with the phosphorylated form of p145βMET (12). For lipocortin-1 such interaction/association sites have not yet been identified. At present we cannot exclude the possibility that the association of lipocortin-1 with the unphosphorylated form of the HGF/SF receptor is due to the great abundance of this protein in this particular cell system. The antisense 21-mer lipocortin-1 oligonucleotide, included with or without an anti-lipocortin-1 antibody in cultured A549 cells, was shown to modulate the synthesis and to inhibit phosphorylation of lipocortin-1 in kinase reactions carried out in vitro and followed by immunoblotting. In parallel, this neutralizing effect of the AsLip and of the LipAb on A549 proliferation suggested that the membrane and the extracellularly associated lipocortin-1 are directly involved as regulators of cellular proliferation. Therefore, the decrease in the availability of lipocortin-1, induced by the presence of AsLip and/or the LipAb, resulting also in decreased phosphorylation of the 38-kDa substrate, is directly correlated with the concomitant decrease in A549 proliferation. It is therefore emerging a functional link between HGF/SF-stimulated A549 proliferation and the phosphorylation state of lipocortin-1. TEA3A1 thymic epithelial cell growth is also regulated by PGE2 release, and when these cells were transfected with antisense annexin I (lipocortin-1) cDNA, the PGE2 production was significantly lower (29). In sense-annexin-I-transfected TEA3A1 cells, the PGE2 release was increased and accomplished by higher levels of cytosolic PLA2 activity (29).

Other studies have reported that stimulation of A549 proliferation with EGF in the presence of lipocortin-1 N-terminal peptide fragments inhibited cell proliferation and suppressed PGE2 release (38). It may be speculated that cellular lipocortin-1 is phosphorylated presumably by ligand-activated receptor tyrosine kinases (20). In case lipocortin-1 levels are increased beyond a level due to exogenously added factor(s) (dexamethasone, N-terminal peptides), the equilibrium is shifted toward the nonphosphorylated fraction that does not fully activate cPLA2 activity.

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