

Reactive Oxygen Species Mediate Met Receptor Transactivation by G Protein-coupled Receptors and the Epidermal Growth Factor Receptor in Human Carcinoma Cells*

Received for publication, March 5, 2004, and in revised form, April 21, 2004
Published, JBC Papers in Press, April 29, 2004, DOI 10.1074/jbc.M402508200

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Cross-communication between the Met receptor tyrosine kinase and the epidermal growth factor receptor (EGFR) has been proposed to involve direct association of both receptors and EGFR kinase-dependent phosphorylation. Here, we demonstrate that in human hepatocellular and pancreatic carcinoma cells the Met receptor becomes tyrosine phosphorylated not only upon EGF stimulation but also in response to G protein-coupled receptor (GPCR) agonists. Whereas specific inhibition of the EGFR kinase activity blocked EGF- but not GPCR agonist-induced Met receptor transactivation, it was abrogated in the presence of a reducing agent or treatment of cells with a NADPH oxidase inhibitor. Both GPCR ligands and EGF are further shown to increase the level of reactive oxygen species within the cell. Interestingly, stimulation of the Met receptor by either GPCR agonists, EGF or its cognate ligand HGF, resulted in release of Met-associated β -catenin and in its Met-dependent translocation into the nucleus, as analyzed by small interfering RNA-mediated knockdown of the Met receptor. Our results provide a new molecular explanation for cell surface receptor cross-talk involving the Met receptor and thereby link the wide diversity of GPCRs and the EGFR to the oncogenic potential of Met signaling in human carcinoma cells.

The proto-oncogene MET encodes the prototypic receptor tyrosine kinase (RTK)¹ for the scatter factor receptor family (1). It has been originally discovered as the product of a human oncogene, *tpo-met* (2, 3), and since then has been recognized as a key mediator of invasive growth in both physiological but also pathophysiological signaling. Stimulation by its cognate ligand hepatocyte growth factor (HGF)/scatter factor induces various biological responses such as cell dissociation, migration, cell scattering, and invasion, but also stimulates cell proliferation or survival depending on the cellular context (4, 5). Whereas Met exerts essential developmental functions (6), deregulated Met receptor signaling because of overexpression, mutation, or

autocrine growth factor loops has been frequently implicated in tumor invasion and metastasis (4) of tumor cells.

The Met RTK consists of a single membrane spanning β -subunit of 145 kDa, which is linked by disulfide bridges to an extracellular α -chain of 50 kDa (1). Upon HGF stimulation two receptor molecules dimerize and cross-phosphorylate each other, thereby creating binding sites for signaling molecules within the cell. By recruiting adaptor proteins such as Gab1 and Grb2 the Met receptor induces activation of phosphatidylinositol 3-kinase/Akt, phospholipase- γ , extracellular signal-regulated kinase 1/2, and the phosphorylation of signal transducers and activators of transcription factors. Moreover, receptor-associated β -catenin was identified as a signaling partner of the Met receptor (7, 8). In the cell, β -catenin exerts a dual function: it allows tight intercellular adhesion, as a member of cell adherence junctions, linking E-cadherin to the cytoskeleton (9) and it translocates into the nucleus to induce gene expression in complex with the transcription factor TCF upon activation of the Wnt-signaling pathway. Independent of Wnt signaling HGF is able to induce β -catenin phosphorylation (10, 11), leading to dissociation of the Met- β -catenin complex, nuclear translocation of β -catenin, and concomitant induction of TCF target genes (7, 12).

Cross-talk between cell surface receptors can occur in several different ways. The most direct mechanism is receptor heterodimerization that is well described for members of the EGFR family (13) or for Met and its relative RON (14). Also less related receptors have been shown to be able to associate such as EGFR and platelet-derived growth factor receptor (15) or Met and semaphorin receptors (16).

In the case of EGFR and Met receptor cross-communication has been reported to occur on different levels: EGFR signal transactivation induces metalloprotease-mediated ectodomain shedding of Met (17) and aberrant EGFR activation elevates Met expression and phosphorylation in thyroid carcinoma cells (18). Moreover, EGFR function has been implicated in HGF-induced hepatocyte proliferation (19), and a possible heterodimer formation of both receptors has been suggested (20, 21).

In addition to RTK cross-talk, also completely unrelated cell surface receptors are able to communicate and influence each other. The prototype for such a pathway is the GPCR-induced EGFR signal transactivation (22). Treatment of cells with GPCR agonists induces phosphorylation of the EGFR by metalloprotease-dependent release of EGF-like ligands (23), thereby coupling GPCRs to EGFR characteristic downstream signaling pathways such as mitogen-activated protein kinases or the phosphatidylinositol 3-kinase/Akt pathway (24). In addition to the EGFR other RTKs have been shown to be acti-

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[§] Supported by a fellowship of the Boehringer Ingelheim Foundation.
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¹ The abbreviations used are: RTK, receptor tyrosine kinase; HGF, hepatocyte growth factor; EGFR, epidermal growth factor receptor; ROS, reactive oxygen species; LPA, lysophosphatidic acid; EGCG, epigallocatechin gallate; DCF-DA, 2',7'-dichlorofluorescein diacetate; PIPES, 1,4-piperazinediethanesulfonic acid; siRNA, small interfering RNA.

vated in response to GPCR stimulation, comprising the insulin-like growth factor-1 receptor (25), Trk receptor (26), platelet-derived growth factor receptor (27, 28), and the vascular endothelial growth factor receptor (29). Receptor cross-talk can also occur in a ligand-independent manner involving for instance, non-receptor tyrosine kinases such as c-Src (26, 30). Moreover, increasing evidence implicated the reactive oxygen species (ROS) as signaling intermediates in RTK activation (31–35). ROS-mediated inhibition of phosphatases results in an equilibrium shift from the non-phosphorylated to the phosphorylated state of the RTK. An increase in ROS levels has been reported not only after RTK but also in response to GPCR stimulation in vascular smooth muscle cells and endothelial cells (36, 37). Interestingly, elevated ROS levels have been associated with the malignant phenotype (38).

Although various cellular sources for ROS have been described (39), plasma membrane-associated NADPH oxidases are considered as the main source of ROS acutely produced upon growth factor or cytokine stimulation (34, 40, 41). Whereas these enzymes have been originally discovered in phagocytes, homologues have also been found in non-phagocytic cells (40). Overexpression of the murine homologue of the NADPH oxidase catalytic subunit was able to induce transformation of NIH-3T3 cells (42). Moreover, overexpression of NADPH oxidase 1 induced tumor formation in mice (43), and recently Arbiser and colleagues (44) reported that tumors induced by NADPH oxidase 1 expression enhanced angiogenesis in a ROS-dependent manner.

Here, we investigated whether and how GPCRs and EGFR could transactivate the proto-oncogenic Met RTK. Our results provide evidence that the Met receptor is transactivated by both GPCRs and EGFR in pancreatic and hepatocellular carcinoma cell lines. This transactivation process involves the acute production of ROS by membrane-bound NADPH oxidases. Interestingly, β -catenin dissociates from transactivated Met receptor and subsequently translocates into the nucleus, an event previously shown to be associated with increased TCF target gene expression and cell motility.

MATERIALS AND METHODS

Reagents—Lysophosphatidic acid (LPA), bradykinin, thrombin, and carbachol were purchased from Sigma. The inhibitors tyrphostin AG1478, epigallocatechin gallate (EGCG), and diphenylethionium chloride were obtained from Alexis Biochemicals. Toxin B and HGF were from Calbiochem. The fluorescent dye 2',7'-dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular Probes.

Cell Culture—All cell lines were obtained from ATCC (American Type Culture Collection, Manassas, VA). The pancreatic carcinoma cell line DAN-G was cultured in RPMI supplemented with 10% fetal bovine serum (Invitrogen) and L-glutamine. HepG2 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum (Invitrogen) and L-glutamine, and HuH7 cells were maintained in Dulbecco's modified Eagle's medium, high glucose, supplemented with 10% fetal bovine serum (Invitrogen), sodium pyruvate, and L-glutamine. Cells were seeded in 10-cm dishes and serum-starved for 24 h prior to stimulation.

Protein Analysis—Prior to lysis, cells grown to 80% confluence were treated with inhibitors and agonists as indicated in the figure legends and then lysed for 10 min on ice in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml aprotinin. Lysates were precleared by centrifugation at 13,000 rpm for 10 min at 4 °C. Precleared lysates were immunoprecipitated using the respective antibodies and 20 μ l of protein A-Sepharose for 4 h at 4 °C. Precipitates were washed three times with 0.5 ml of HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$), suspended in 2 \times SDS sample buffer, boiled for 3 min, and subjected to gel electrophoresis. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membrane. Western blots were performed according to standard methods. The

antibody against human EGFR (108.1) has been characterized before (23). Met was precipitated using the polyclonal C-28 antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphotyrosine was detected with the 4G10 monoclonal antibody (Ubi). β -Catenin was detected with a monoclonal antibody purchased from UBI (Lake Placid, NY). Data shown are representative for three independent experiments. Quantification was done using the Fuji LAS1000 imaging system.

Detection of ROS by Flow Cytometric Analysis—Cells were seeded in 6-well dishes and grown for 48 h. Prior to stimulation cells were preincubated with 20 μ M DCF-DA for 30 min and subsequently stimulated as indicated for 15 min. After trypsinization, cells were centrifuged at 800 \times g and resuspended in phosphate-buffered saline with propidium iodide. Cells were immediately analyzed on a BD Biosciences FACScalibur flow cytometer.

Differential Detergent Fractionation—Differential detergent fractionation was carried out as described before (60). Cells were pretreated with inhibitors as indicated in the figure legends. Following stimulation, cells were washed in phosphate-buffered saline, and a cytosolic-enriched fraction was obtained using a digitonin buffer (0.01% digitonin, 10 mM PIPES, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl_2 , 5 mM EDTA) and centrifugation at 480 \times g. The supernatant was carefully removed as the cytosolic-enriched fraction. The membrane-enriched fraction was obtained by resuspending the pellet in Triton X-100 buffer (0.5% Triton X-100, 10 mM PIPES, pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl_2 , 3 mM EDTA), centrifugation at 5000 \times g for 10 min, and careful removal of the supernatant. The pellet obtained was resuspended in Tween 40/DOC buffer (1% Tween 40, 0.5% deoxycholate, 10 mM PIPES, pH 7.4, 10 mM NaCl, 1 mM MgCl_2). To break up nuclei, 20 strokes in a Dounce homogenizer were applied followed by ultrasonication and centrifugation at 6780 \times g. The supernatant was collected as the nuclei-enriched fraction. Protein concentration was determined using the BCA protein assay kit (Pierce), and equal amounts of protein were subjected to gel electrophoresis and Western blotting.

MET Modulation—MET expression was down-modulated by siRNA using the oligo ACUCUAGAUGCUCAGACUUTT cloned in a lentiviral vector under the control of the H1 promoter. Infections were performed as described (61). As a negative control, cells were infected with a lentivirus containing the same oligo mutated in three bases (AGUCUACAUGCUCACACUU) or an oligo specific for GFP sequences. After infection, cells were kept in culture for 72 h and then extracted with boiling Laemmli buffer. Equal amounts of proteins (evaluated by BCA; Pierce) were loaded in each lane. Blots were probed with antibodies to human MET (C12, Santa Cruz).

Statistical Analysis—For statistical analysis, Student's *t* test was used to compare data between two groups. Values are expressed as mean \pm S.D. of three independent experiments. *p* < 0.05 was considered statistically significant.

RESULTS

GPCR and EGFR Stimulation Induce Met Receptor Phosphorylation—Because we were interested in investigating whether the Met receptor can function as a downstream signaling partner of different membrane receptors, therefore acting as a point of convergence for heterogeneous signaling pathways, we used immunoblot analysis to assess the phosphorylation state of this receptor in response to treatment with different GPCR agonists. As shown in Fig. 1, Met receptor tyrosine phosphorylation was rapidly induced in response to different GPCR ligands in the pancreatic carcinoma cell line DAN-G as well as in the hepatocellular carcinoma cell lines HepG2 and HuH7 (Fig. 1A). This activation occurred within 3 min and declined after 7–15 min as shown for DAN-G and HepG2 cells (Fig. 1B), demonstrating a rapid and transient transactivation of the Met receptor. Interestingly, not only GPCR ligands but also EGF induced stimulation of the Met receptor (Fig. 1A). Met phosphorylation observed in these conditions was similar in terms of intensity and timing to that elicited on stimulation with 4 ng/ml HGF (Fig. 2A).

GPCR-induced Met Transactivation Is Independent from EGFR Kinase Activity—The so far best characterized receptor cross-talk mechanism is GPCR-induced EGFR signal transactivation. The mechanism was shown to involve the protease-

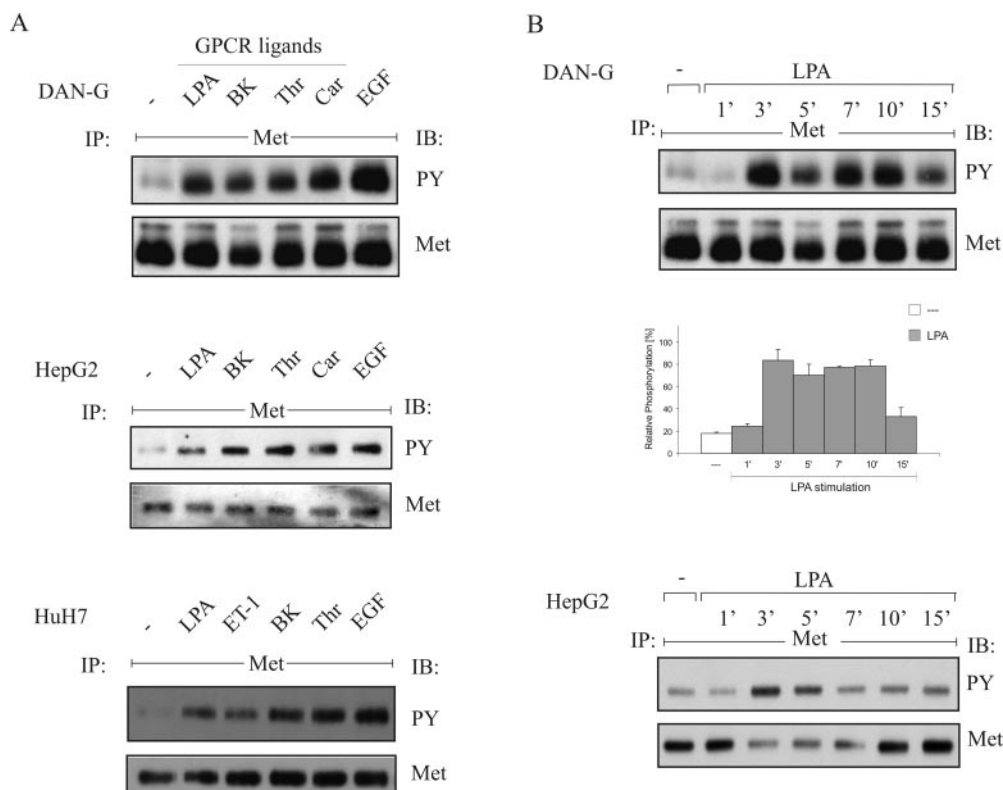


FIG. 1. GPCR agonists and EGF induce Met receptor transactivation. *A*, cells were serum-starved for 24 h, treated with lysophosphatidic acid (LPA, 10 μ M), bradykinin (BK, 2 μ M), thrombin (Thr, 2 units/ml), carbachol (Car, 10 μ M), endothelin (ET-1, 200 nM), or EGF (4 ng/ml) as indicated for 3 min. Following immunoprecipitation (IP) of cell extracts with anti-Met antibody, proteins were immunoblotted (IB) with anti-phosphotyrosine antibody (PY) and re-probed with anti-Met antibody. *B*, cells were stimulated with LPA (10 μ M) for the indicated time periods. Cell lysates were treated as described under *A*. Quantification of DAN-G immunoblots was carried out using the Fuji LAS1000 imaging system.

dependent shedding of an EGF-like ligand precursor. As previous reports have described the association between Met and EGFR and the phosphorylation of Met by the EGFR (20, 21), we addressed the question whether GPCR-Met transactivation requires EGFR kinase activity. If so, the Met receptor would behave as a direct signaling partner of the EGFR.

To address this question we used the EGFR kinase-specific inhibitor AG1478 to block EGFR-dependent phosphorylation events. As demonstrated in Fig. 2, pretreatment of cells with AG1478 blocked GPCR-induced EGFR signal transactivation (22, 23) but did not affect GPCR agonist-induced phosphorylation of the Met receptor. This finding implicates that EGFR activity is dispensable for GPCR-induced Met receptor transactivation (Fig. 2, *A* and *B*).

As expected, Met transactivation by EGF was abrogated by preincubation with AG1478 (Fig. 2, *A* and *B*), as the EGFR kinase is mandatory to transmit the EGF-stimulated signal, regardless of direct association or an indirect communication with the Met receptor. In contrast to the report by Jo and colleagues (20) we were unable to detect direct binding between Met and EGFR in hepatocellular and pancreatic carcinoma cells (data not shown).

GPCR- and EGF-stimulated ROS Production in Carcinoma Cell Lines—Inactivation of tyrosine phosphatases by growth factor-induced production of ROS has been suggested to account for the activation of RTKs (31–34, 41). Because Met receptor transactivation occurred rapidly and transiently, we asked whether Met receptor activation is mediated by a transient increase of ROS levels. We used the fluorescence dye DCF-DA to detect ROS production. DCF-DA is a membrane-permeable non-fluorescent compound, which is de-esterified inside the cell. Subsequent oxidation of dichlorofluorescein generates the fluorescent dye itself. Flow cytometric analysis of

cells treated with DCF-DA and subsequent stimulation with EGF or the GPCR ligands LPA and thrombin clearly demonstrated an increase of reactive oxygen species within the cell (Fig. 3, *A* and *B*).

Further preincubation of DAN-G and HepG2 cells with the reducing agent EGCG interfered with EGF- and GPCR-induced Met receptor phosphorylation, underlining the involvement of ROS in this signaling pathway. On the other hand, EGCG treatment did not affect EGFR signal transactivation (Fig. 4, *A* and *B*). Taken together, these results demonstrate that ROS are critically involved in Met receptor transactivation.

Met Receptor Transactivation Requires NADPH Oxidase Activity—Recent lines of research implicated membrane-bound NADPH oxidases in the growth factor stimulated production of ROS (reviewed in Refs. 34, 41, and 45). To investigate whether these NADPH oxidase enzymes are involved in Met receptor transactivation, we used the NADPH oxidase-specific inhibitor diphenyleneiodonium chloride to interfere with NADPH oxidase-dependent ROS production and analyzed Met receptor phosphorylation by immunoblot analysis. As shown in Fig. 5, blockade of NADPH oxidase function interfered with both GPCR- and EGF-induced Met phosphorylation in HepG2 and DAN-G cells, whereas EGFR signal transactivation by GPCRs was not affected by diphenyleneiodonium chloride treatment (Fig. 5, *A* and *B*).

The small GTPase Rac has been demonstrated to be one of the cytosolic components of the NADPH oxidase enzyme complex and has been implicated in the regulation of NADPH oxidases activity (46). Moreover, Rac has been shown as a downstream signaling target of both GPCRs and RTKs. Therefore we used the bacterial Toxin B, which specifically blocks GTPases of the Rac, Rho, and Cdc42 families, to interfere with

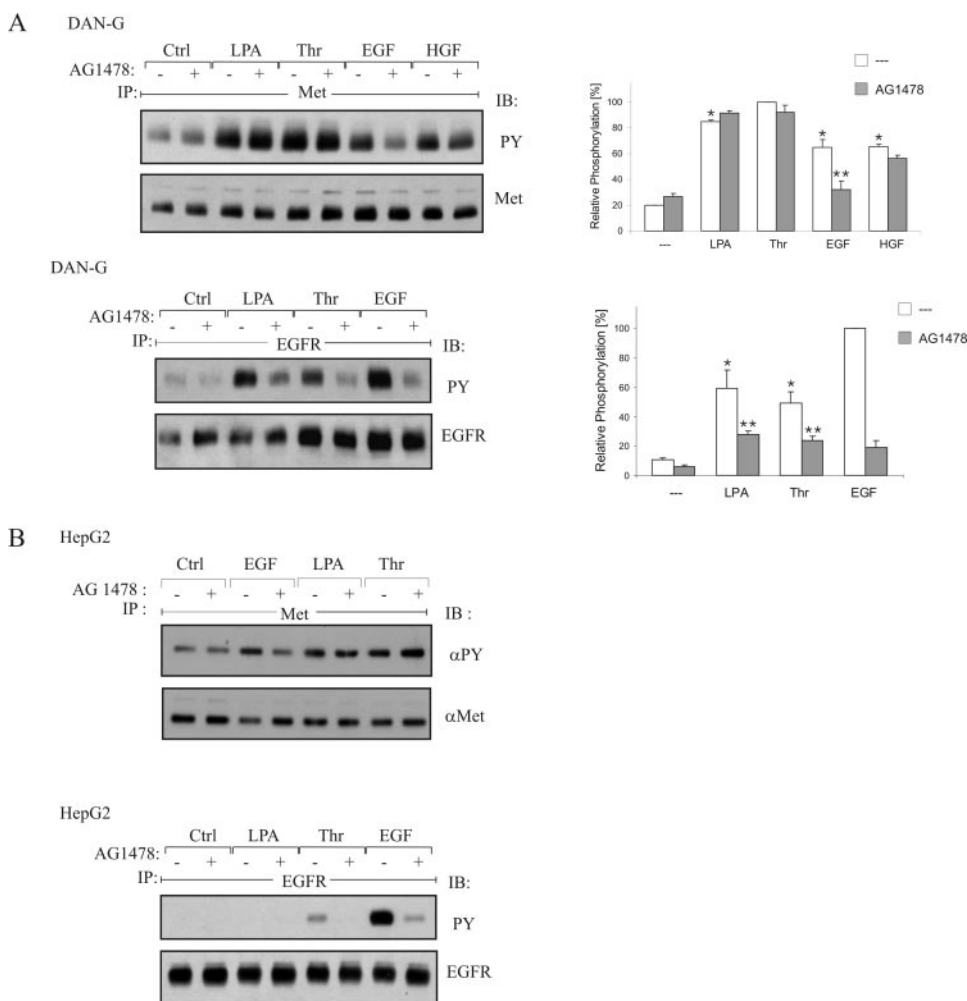


FIG. 2. GPCR-induced Met transactivation is independent of EGFR kinase activity. *A* and *B*, DAN-G and HepG2 cells were pretreated with AG1478 (250 nM) or an equal volume of empty vehicle (Me₂SO) for 20 min and subsequently stimulated with LPA (10 μM), Thr (2 units/ml), EGF (4 ng/ml), or HGF (4 ng/ml) as positive controls for 3 min. Following immunoprecipitation of cell extracts with anti-Met antibody, proteins were immunoblotted with anti-phosphotyrosine antibody and reprobbed with anti-Met antibody. EGFR phosphorylation content was analyzed by immunoprecipitation of cell extracts with anti-EGFR antibody and immunoblotting with anti-phosphotyrosine. The same filters were reprobbed with anti-EGFR antibody. Quantification of DAN-G immunoblots was carried out using the Fuji LAS1000 imaging system. *, $p < 0.01$ for stimulation *versus* control; **, $p < 0.05$ for inhibition *versus* stimulation.

Rac function (47). Indeed, preincubation with Toxin B abrogated both GPCR- and EGF-induced Met transactivation (Fig. 5, *C* and *D*, upper panel), whereas EGFR signal transactivation was not influenced (Fig. 5, *C* and *D*, lower panel). Together, these data suggest that NADPH oxidase activity is indeed required for Met transactivation.

Met Transactivation Induces Dissociation of the β -Catenin-Met Receptor Complex in HuH7 and DAN-G Cells— β -Catenin has been shown to be constitutively associated with the Met receptor in hepatoma and hepatocellular carcinoma cells (8, 48). Immunoprecipitation of Met from untreated HuH7 hepatocellular carcinoma cells co-precipitated constitutively tyrosine phosphorylated β -catenin (Fig. 6A). Stimulation of Met transactivation by both GPCR ligands and EGF induced the dissociation of this β -catenin-Met receptor complex (Fig. 6A).

To analyze the translocation of β -catenin in response to Met transactivation we used the differential detergent fractionation technique to prepare cytoplasmic-, membrane-, and nuclei-enriched fractions. Fig. 6B demonstrates that stimulation of HuH7 as well as DAN-G cells with LPA, thrombin, EGF, or HGF induced translocation of β -catenin from the membrane to the cytoplasmic fraction and the increase of β -catenin in the nuclei-enriched fraction. To investigate whether the β -catenin translocation in response to GPCR agonists or EGF depends on

the Met receptor, we used a siRNA approach to knockdown the Met receptor protein level. As shown in Fig. 6C, infection of HuH7 cells with a siRNA expression vector induced efficient knockdown of Met. Stimulation of these cells with LPA, thrombin, EGF, or HGF failed to induce nuclear accumulation of β -catenin (Fig. 6D). Interestingly, knockdown of the Met receptor resulted in a slightly higher basal level of nuclear β -catenin in unstimulated cells compared with control-infected cells.

DISCUSSION

Cross-talk between cell surface receptors has been early recognized as a crucial signaling mechanism to expand the cellular communication network. Related RTKs such as members of the EGFR family are capable of forming heterodimers thereby affecting downstream signaling pathways (13). A further layer of complexity was added by the discovery of the heterogeneous GPCR-RTK cross-talk mechanism with EGFR signal transactivation serving as the prototypic inter-receptor signaling pathway (22, 23). Because in addition to the above cross-communication, pathways involving integrins and cytokine receptors have also been reported (49–51), increasing interest is now focused on these heterogeneous inter-receptor networks.

Here, we provide experimental evidence that the EGFR

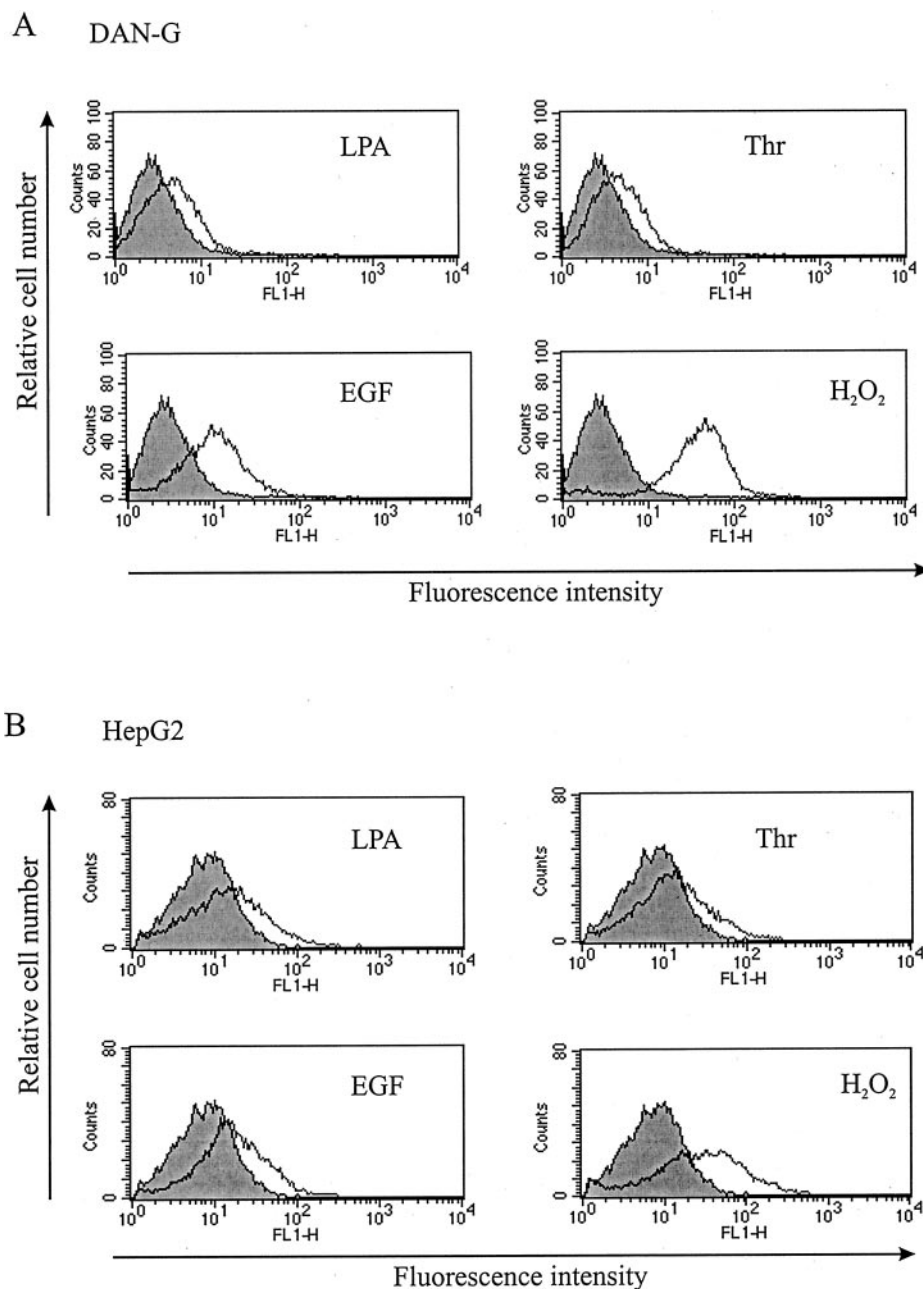


FIG. 3. GPCR agonists and EGF increase ROS production in DAN-G and HepG2 cells. A and B, cells were seeded into 6-well dishes, grown for 48 h, and incubated with DCF-DA (20 μ M) for 30 min. After stimulation with LPA (10 μ M), Thr (2 units/ml), EGF (4 ng/ml), or hydrogen peroxide (H₂O₂, 500 μ M) as positive controls for 15 min, cells were collected, resuspended in phosphate-buffered saline supplemented with propidium iodide, and immediately analyzed by flow cytometric analysis.

transactivates the Met RTK in human carcinoma cells. Previous investigations demonstrated cross-talk between these receptors on the transcriptional level and Met ectodomain shedding in response to EGFR activation (17, 18). Direct association of Met with and phosphorylation by the EGFR has been suggested as the underlying mechanism of this cross-communication (20). EGF- and TGF- α -induced Met phosphorylation and Met-EGFR co-precipitation were restricted to A431 cells that overexpress EGFR because of gene amplification. However, in hepatocellular carcinoma cell lines expressing moderate levels of both receptors, the authors could neither detect transactivation of Met nor coprecipitation of Met and EGFR (20). Because we did neither find association of Met and EGFR in hepatocellular and pancreatic carcinoma cells, we investigated whether an alternative mechanism than heterodimerization might account for Met receptor transactivation.

Using a flow cytometric analysis of cells stained with the redox-sensitive fluorescent dye DCF-DA we could show that reactive oxygen species are generated in response to EGF treat-

ment (Fig. 3). The critical role of ROS in this cross-talk mechanism was further corroborated by the finding that preincubation with the reducing agent EGCG interferes with Met receptor transactivation (Fig. 4). Whereas different cellular sources for ROS have been reported (39), our data provide evidence for the involvement of plasma membrane-bound NADPH oxidases (Figs. 4, A and B; and 5, A–D). These enzymes have previously been implicated in the acute and rapid production of ROS in response to growth factor treatment (34, 39, 41, 45). Moreover, the finding that inhibition of the small GTPases Rac, Rho, and Cdc42 by Toxin B interfered with Met transactivation does also point toward an involvement of NADPH oxidases enzymes, as Rac has been shown to regulate the activity of the NADPH oxidases enzyme complex.

In addition to EGF, also stimulation of cells with GPCR ligands such as LPA, bradykinin, thrombin, or carbachol induced the rapid and transient phosphorylation of the Met receptor (Fig. 1). In analogy to EGF-induced Met transactivation we could demonstrate that GPCR-induced Met phosphoryla-

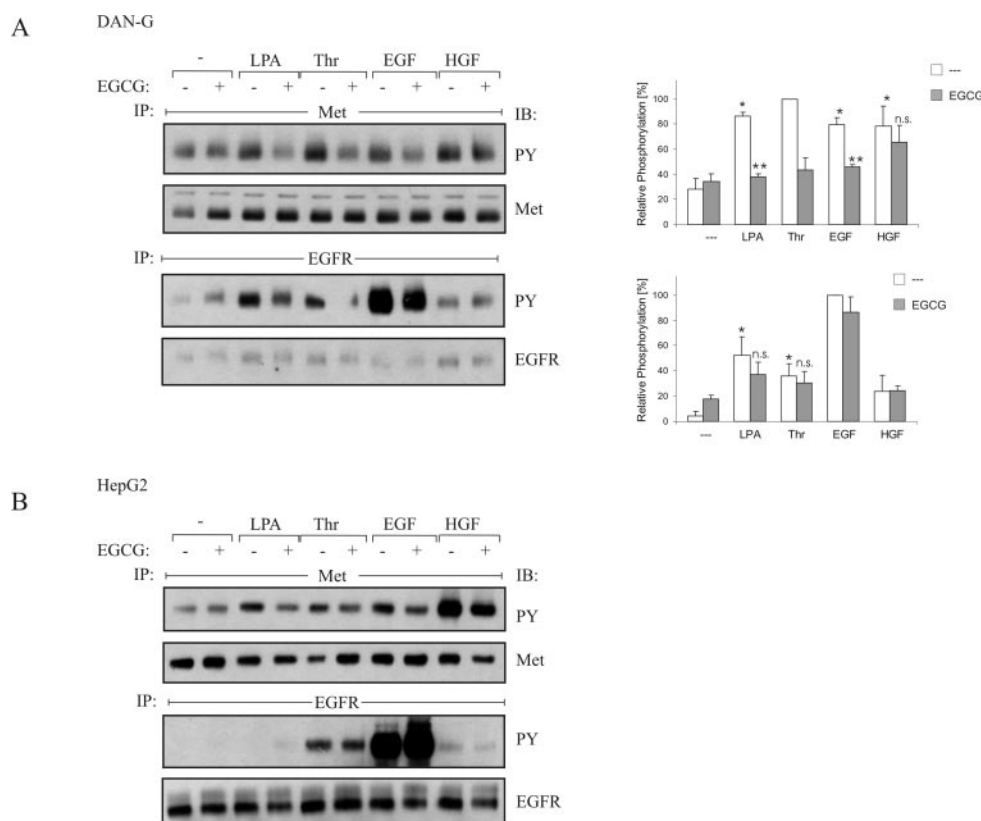


FIG. 4. EGCG abolishes Met receptor transactivation. Cells were preincubated with EGCG (40 μ M) for 60 min. Following stimulation as described in the legend to Fig. 2, Met (A and B, upper panels) or EGFR (A and B, lower panels), respectively, were immunoprecipitated (IP) and analyzed for their phosphotyrosine content. The same filters were stripped and reprobed for Met and EGFR, respectively. Quantification of DAN-G immunoblots was carried out using the Fuji LAS1000 imaging system. *, $p < 0.007$ for stimulation versus control; **, $p < 0.01$ for inhibition versus stimulation. n.s. is not significant for inhibition versus stimulation.

tion critically depends on the production of ROS by NADPH oxidases (Figs. 3, A and B; 4, A and B; and 5, A–D). Flow cytometric analysis showed that stimulation with LPA, thrombin, or EGF induced a moderate shift toward higher fluorescence compared with stimulation with the positive control H_2O_2 . This observation can be anticipated as the ROS production induced by physiologic stimuli in epithelial cells should be strictly limited in its duration and localization, which are in this case distinct microdomains at the plasma membrane. In consequence, only a fraction of the fluorescent dye distributed throughout the cell can be oxidized in response to growth factor treatment. The increase in ROS production corresponds to a moderate increase of Met receptor phosphorylation, which is equivalent to stimulation with 4 ng/ml HGF (Fig. 2). Interestingly, the level of receptor phosphorylation is comparable with the level of EGFR transactivation reached by GPCR ligands.

Very recently, Pai and colleagues (21) reported that prostaglandin E_2 is capable to induce Met receptor phosphorylation in colorectal carcinoma cells. They suggested an indirect mechanism involving the EGFR, because preincubation with the EGFR kinase-specific inhibitor AG1478 abolished Met activation (21). Interestingly, EGF treatment did not significantly enhance Met phosphorylation. However, treatment of hepatocellular and pancreatic carcinoma cells with AG1478 did not affect Met phosphorylation in response to GPCR agonists, suggesting that GPCR-induced Met phosphorylation does not depend on EGFR kinase activity in these cellular systems. Because the EGFR itself is able to induce ROS production (31), in some cellular systems EGFR signal transactivation might be used by GPCRs that are unable to directly stimulate the production of reactive oxygen intermediates to induce ROS-dependent Met transactivation.

The involvement of ROS in Met transactivation is corroborated by the previous findings that platelet-derived growth factor induces ROS production in HepG2 cells (52). The same result has been obtained in A431 cells in response to EGF treatment (31). Because in colorectal carcinoma cells that have been used in the study by Pai and colleagues (21), functional components of NADPH oxidases enzymes have been found (53, 54), the results presented herein can provide a molecular explanation for activation of Met in response to heterogeneous stimuli in the cellular systems reported by Stolz and co-workers (20) and Pai *et al.* (21). ROS have been previously implicated in receptor cross-talk mechanisms, in particular in endothelial or vascular smooth muscle cells, such as angiotensin II-induced EGFR transactivation (33). Our results extend this finding and demonstrate this functional correlation of GPCR and Met cross-talk in transformed epithelial cells of hepatocellular and pancreatic carcinomas.

Previous reports demonstrated the association between the Met receptor and β -catenin (7, 8, 12). Interestingly, we could show that in the hepatocellular carcinoma cell line HuH7 and in the pancreatic carcinoma cell line DAN-G not only direct Met activation by HGF but also Met transactivation by GPCR agonists and EGF stimulates the nuclear translocation of β -catenin. Nuclear β -catenin has been associated with enhanced transcription of TCF target genes that are involved in epithelial to mesenchymal transition and the development of an invasive phenotype (9, 55, 56). The Met receptor has been shown to be instrumental to invasive growth in both the physiological and pathophysiological signaling context (4). Interestingly, previous reports implicated co-expression of Met and EGFR in tumor progression and development in gastrinoma and hepatocellular carcinoma. In the latter, overexpression of

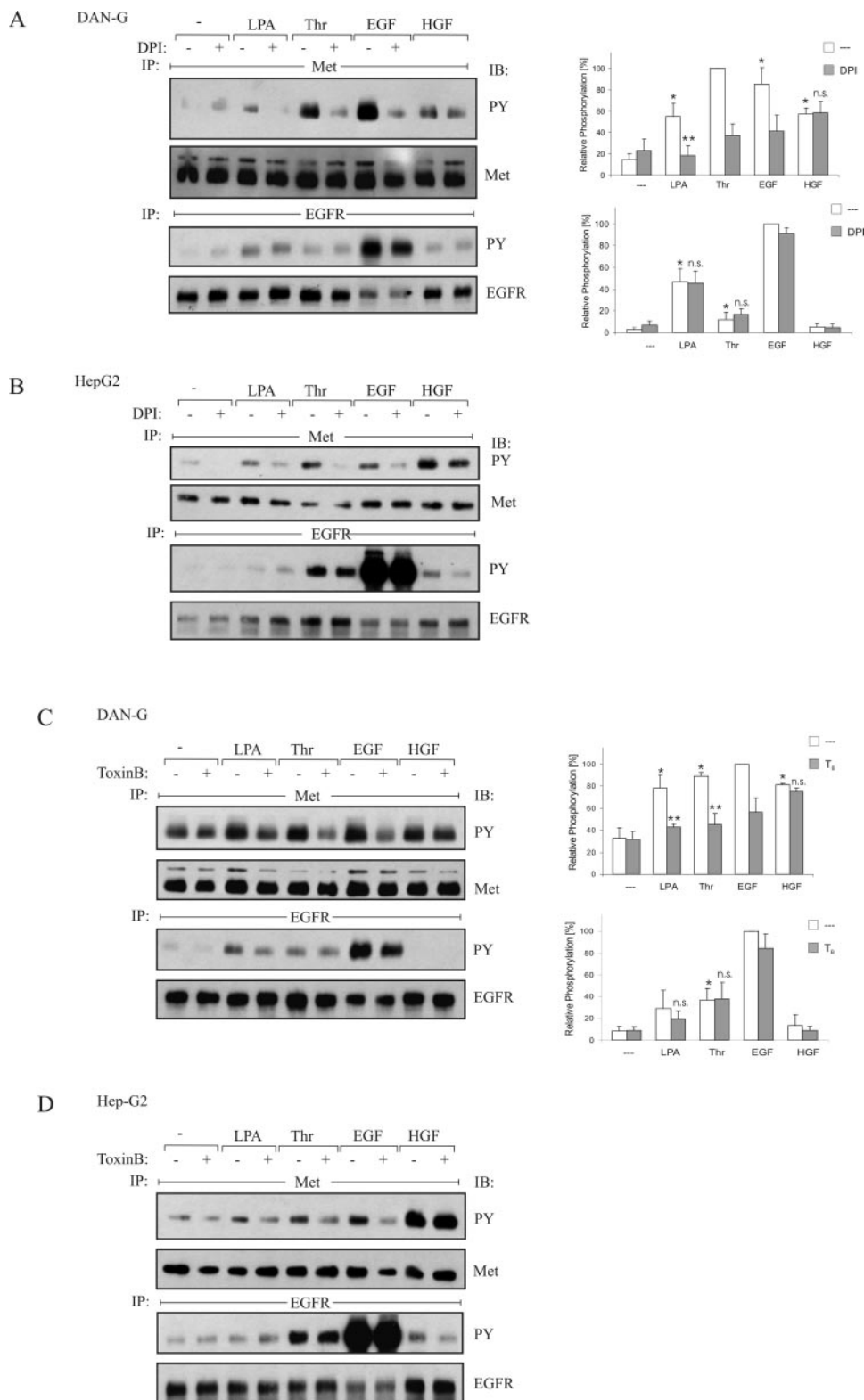


FIG. 5. ROS production depends on NADPH oxidase activity. *A* and *B*, following preincubation with diphenyleneiodonium chloride (DPI) (10 μ M) for 30 min, cells were stimulated as indicated and Met (upper panel) or EGFR (lower panel) were analyzed for their phosphotyrosine content by immunoblot analysis. *C* and *D*, cells were pretreated with Toxin B (2 ng/ml) for 2 h, stimulated as indicated, and analyzed as described under *A* and *B*. Quantification of DAN-G immunoblots was carried out using the Fuji LAS1000 imaging system. *, $p < 0.04$ for stimulation versus control; **, $p < 0.01$ for inhibition versus stimulation. n.s. is not significant for inhibition versus stimulation.

both Met and the EGFR is associated with increased tumor size, tumor stage, lymph node metastasis, poor prognosis, and reduced survival (57). Daveau *et al.* (58) reported the presence of enhanced levels of mRNA for both receptors, in particular in poorly differentiated tumors and in patients with early tumor

recurrence. Noteworthy, Miura and colleagues (59) demonstrated that ROS potentiated the invasive activity of hepatoma cells by the autocrine/paracrine loop of HGF.

The Met receptor and its ligand HGF are able to induce complex biological responses such as branching morphogene-

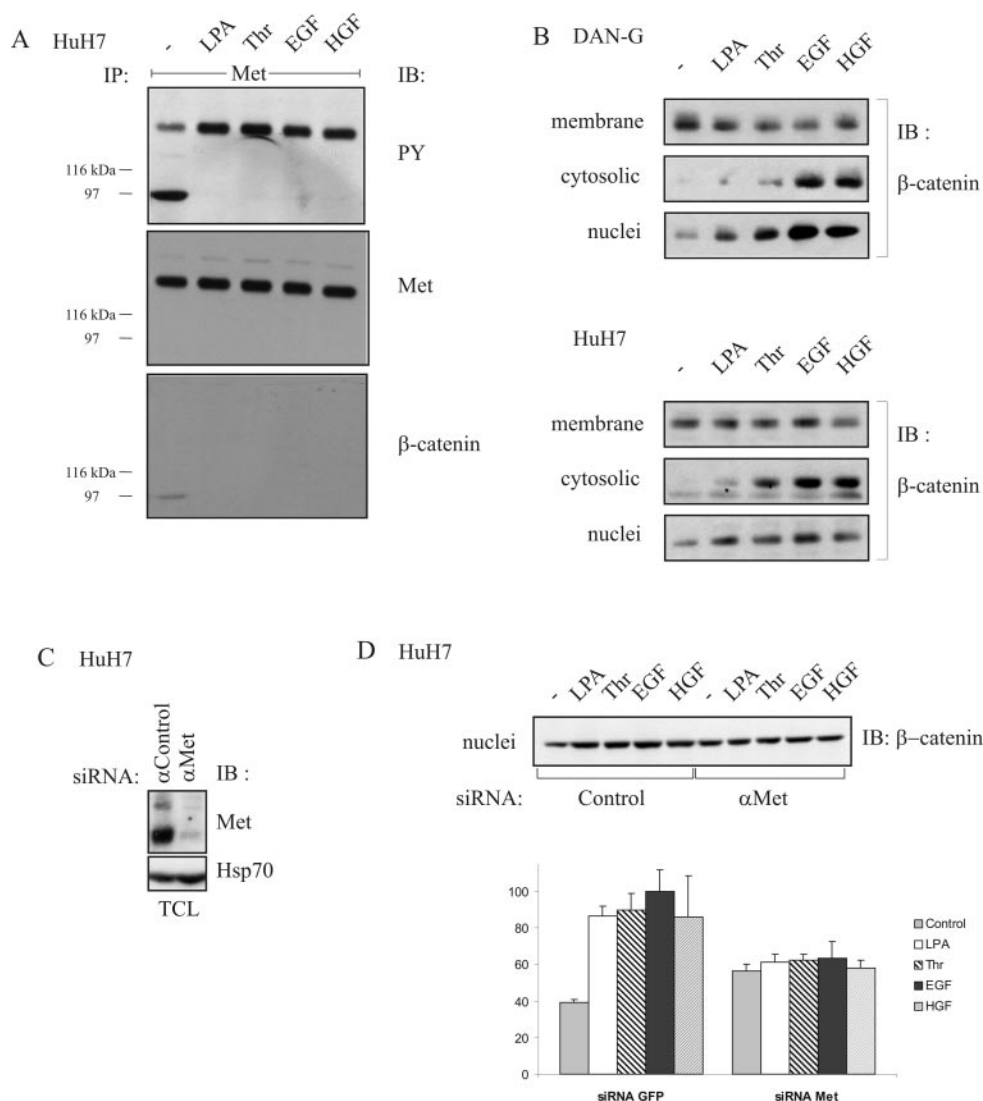


FIG. 6. Met transactivation induces nuclear translocation of β -catenin. *A*, HuH7 cells were serum-starved for 24 h and stimulated as indicated. Following immunoprecipitation (IP) of the Met receptor, filters were probed for phosphotyrosine content, Met, or β -catenin. *B*, DAN-G or HuH7 cells were stimulated as depicted and subsequently cytoplasmic-, membrane- and nucleic-enriched fractions were isolated by differential detergent fractionation (DDF) as described under "Materials and Methods." Equal amounts of total lysates were analyzed for β -catenin content by immunoblot analysis. *C*, HuH7 cells infected with the Met-specific or a control siRNA were lysed and Met receptor contents was assessed by immunoblot analysis on the total lysate. *D*, HuH7 cells were infected with siRNA expression vectors as previously described. Control- and Met siRNA-infected cells were treated as described under *B*. Experiments were performed in triplicate and quantified using the Fuji LAS1000 imaging system.

sis. However, EGF is not able to trigger this effect, although the herein discussed signaling pathway links EGFR and Met receptor signaling. There are two major differences between Met receptor stimulation with its cognate ligand HGF and transactivation of Met. First, whereas exogenous HGF stimulation induces the strongest receptor phosphorylation after 10–15 min, Met receptor transactivation by GPCRs or EGFR peaks after 3–5 min, indicating a different time course of receptor phosphorylation. Both a short activity of ROS or subsequent phosphatase activation might lead to this shortened Met stimulation compared with exogenous HGF treatment. Second, the level of phosphorylation in response to Met receptor transactivation is weaker compared with the phosphorylation induced by HGF concentrations of 20 ng/ml, which are used to induce for example, branching morphogenesis.

Taken together, our results provide a molecular mechanism for EGFR-dependent transactivation of the Met receptor and extend this concept to cross-talk involving GPCRs and Met. With respect to the outstanding role of the Met receptor in tumor invasion, these findings are of special significance for the

understanding of cancer progression and metastasis in response to heterogeneous growth factor stimulation involving the Met receptor.

Acknowledgment—We thank M. Buschbeck for critical revision of the manuscript.

REFERENCES

- Giordano, S., Ponzetto, C., Di Renzo, M. F., Cooper, C. S., and Comoglio, P. M. (1989) *Nature* **339**, 155–156
- Cooper, C. S., Park, M., Blair, D. G., Tainsky, M. A., Huebner, K., Croce, C. M., and Vande Woude, G. F. (1984) *Nature* **311**, 29–33
- Park, M., Dean, M., Kaul, K., Braun, M. J., Gonda, M. A., and Vande Woude, G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6379–6383
- Trusolino, L., and Comoglio, P. M. (2002) *Nat. Rev. Cancer* **2**, 289–300
- Zhang, Y. W., and Vande Woude, G. F. (2003) *J. Cell. Biochem.* **88**, 408–417
- Birchmeier, C., and Gherardi, E. (1998) *Trends Cell Biol.* **8**, 404–410
- Monga, S. P., Mars, W. M., Padiaditakis, P., Bell, A., Mule, K., Bowen, W. C., Wang, X., Zarnegar, R., and Michalopoulos, G. K. (2002) *Cancer Res.* **62**, 2064–2071
- Hiscox, S., and Jiang, W. G. (1999) *Biochem. Biophys. Res. Commun.* **261**, 406–411
- Conacci-Sorrell, M., Zhurinsky, J., and Ben-Ze'ev, A. (2002) *J. Clin. Invest.* **109**, 987–991
- Hiscox, S., and Jiang, W. G. (1999) *Anticancer Res.* **19**, 509–517
- Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K.,

- Kitamura, N., Takeichi, M., and Ito, F. (1994) *Cell Adhes. Commun.* **1**, 295–305
12. Muller, T., Bain, G., Wang, X., and Papkoff, J. (2002) *Exp. Cell Res.* **280**, 119–133
13. Yarden, Y., and Sliwkowski, M. X. (2001) *Nat. Rev. Mol. Cell Biol.* **2**, 127–137
14. Follenzi, A., Bakovic, S., Gual, P., Stella, M. C., Longati, P., and Comoglio, P. M. (2000) *Oncogene* **19**, 3041–3049
15. Saito, Y., Haendeler, J., Hojo, Y., Yamamoto, K., and Berk, B. C. (2001) *Mol. Cell Biol.* **21**, 6387–6394
16. Giordano, S., Corso, S., Conrotto, P., Artigiani, S., Gilestro, G., Barberis, D., Tamagnone, L., and Comoglio, P. M. (2002) *Nat. Cell Biol.* **4**, 720–724
17. Nath, D., Williamson, N. J., Jarvis, R., and Murphy, G. (2001) *J. Cell Sci.* **114**, 1213–1220
18. Bergstrom, J. D., Westermark, B., and Heldin, N. E. (2000) *Exp. Cell Res.* **259**, 293–299
19. Scheving, L. A., Stevenson, M. C., Taylormore, J. M., Traxler, P., and Russell, W. E. (2002) *Biochem. Biophys. Res. Commun.* **290**, 197–203
20. Jo, M., Stolz, D. B., Esplen, J. E., Dorko, K., Michalopoulos, G. K., and Strom, S. C. (2000) *J. Biol. Chem.* **275**, 8806–8811
21. Pai, R., Nakamura, T., Moon, W. S., and Tarnawski, A. S. (2003) *FASEB J.* **17**, 1640–1647
22. Daub, H., Weiss, F. U., Wallasch, C., and Ullrich, A. (1996) *Nature* **379**, 557–560
23. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) *Nature* **402**, 884–888
24. Gschwind, A., Prenzel, N., and Ullrich, A. (2002) *Cancer Res.* **62**, 6329–6336
25. Rao, G. N., Delafontaine, P., and Runge, M. S. (1995) *J. Biol. Chem.* **270**, 27871–27875
26. Lee, F. S., and Chao, M. V. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3555–3560
27. Linseman, D. A., Benjamin, C. W., and Jones, D. A. (1995) *J. Biol. Chem.* **270**, 12563–12568
28. Herrlich, A., Daub, H., Knebel, A., Herrlich, P., Ullrich, A., Schultz, G., and Gudermann, T. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8985–8990
29. Thuringer, D., Maulon, L., and Frelin, C. (2002) *J. Biol. Chem.* **277**, 2028–2032
30. Tanimoto, T., Jin, Z. G., and Berk, B. C. (2002) *J. Biol. Chem.* **277**, 42997–43001
31. Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B., and Rhee, S. G. (1997) *J. Biol. Chem.* **272**, 217–221
32. Sundaresan, M., Yu, Z. X., Ferrans, V. J., Irani, K., and Finkel, T. (1995) *Science* **270**, 296–299
33. Ushio-Fukai, M., Griendling, K. K., Becker, P. L., Hilenski, L., Halleran, S., and Alexander, R. W. (2001) *Arterioscler. Thromb. Vasc. Biol.* **21**, 489–495
34. Rhee, S. G., Bae, Y. S., Lee, S. R., and Kwon, J. (2000) <http://stke.sciencemag.org/cgi/content/full/sigtrans;2000/53/PE1>
35. Saito, Y., and Berk, B. C. (2001) *J. Mol. Cell Cardiol.* **33**, 3–7
36. Patterson, C., Ruef, J., Madamanchi, N. R., Barry-Lane, P., Hu, Z., Horaist, C., Ballinger, C. A., Brasier, A. R., Bode, C., and Runge, M. S. (1999) *J. Biol. Chem.* **274**, 19814–19822
37. Holland, J. A., Meyer, J. W., Chang, M. M., O'Donnell, R. W., Johnson, D. K., and Ziegler, L. M. (1998) *Endothelium* **6**, 113–121
38. Szatrowski, T. P., and Nathan, C. F. (1991) *Cancer Res.* **51**, 794–798
39. Thannickal, V. J., and Fanburg, B. L. (2000) *Am. J. Physiol.* **279**, L1005–L1028
40. Lambeth, J. D. (2002) *Curr. Opin. Hematol.* **9**, 11–17
41. Finkel, T. (2003) *Curr. Opin. Cell Biol.* **15**, 247–254
42. Suh, Y. A., Arnold, R. S., Lassegue, B., Shi, J., Xu, X., Sorescu, D., Chung, A. B., Griendling, K. K., and Lambeth, J. D. (1999) *Nature* **401**, 79–82
43. Arnold, R. S., Shi, J., Murad, E., Whalen, A. M., Sun, C. Q., Polavarapu, R., Parthasarathy, S., Petros, J. A., and Lambeth, J. D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 5550–5555
44. Arbiser, J. L., Petros, J., Klafter, R., Govindajaran, B., McLaughlin, E. R., Brown, L. F., Cohen, C., Moses, M., Kilroy, S., Arnold, R. S., and Lambeth, J. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 715–720
45. Finkel, T. (2000) *FEBS Lett.* **476**, 52–54
46. Sundaresan, M., Yu, Z. X., Ferrans, V. J., Sulciner, D. J., Gutkind, J. S., Irani, K., Goldschmidt-Clermont, P. J., and Finkel, T. (1996) *Biochem. J.* **318**, 379–382
47. Schiavo, G., and van der Goot, F. G. (2001) *Nat. Rev. Mol. Cell Biol.* **2**, 510–537
48. Herynk, M. H., Tsan, R., Radinsky, R., and Gallick, G. E. (2003) *Clin. Exp. Metastasis* **20**, 291–300
49. Moro, L., Venturino, M., Bozzo, C., Silengo, L., Altruda, F., Beguinot, L., Tarone, G., and Defilippi, P. (1998) *EMBO J.* **17**, 6622–6632
50. Trusolino, L., Bertotti, A., and Comoglio, P. M. (2001) *Cell* **107**, 643–654
51. Yamauchi, T., Ueki, K., Tobe, K., Tamemoto, H., Sekine, N., Wada, M., Honjo, M., Takahashi, M., Takahashi, T., Hirai, H., Tushima, T., Akanuma, Y., Fujita, T., Komuro, I., Yazaki, Y., and Kadowaki, T. (1997) *Nature* **390**, 91–96
52. Bae, Y. S., Sung, J. Y., Kim, O. S., Kim, Y. J., Hur, K. C., Kazlauskas, A., and Rhee, S. G. (2000) *J. Biol. Chem.* **275**, 10527–10531
53. Kikuchi, H., Hikage, M., Miyashita, H., and Fukumoto, M. (2000) *Gene (Amst.)* **254**, 237–243
54. Perner, A., Andresen, L., Pedersen, G., and Rask-Madsen, J. (2003) *Gut* **52**, 231–236
55. Behrens, J. (2000) *Ann. N. Y. Acad. Sci.* **910**, 21–33, 33–35
56. Mareel, M., and Leroy, A. (2003) *Physiol. Rev.* **83**, 337–376
57. Peghini, P. L., Iwamoto, M., Raffeld, M., Chen, Y. J., Goebel, S. U., Serrano, J., and Jensen, R. T. (2002) *Clin. Cancer Res.* **8**, 2273–2285
58. Daveau, M., Scotte, M., Francois, A., Coulouarn, C., Ros, G., Tallet, Y., Hiron, M., Hellot, M. F., and Salier, J. P. (2003) *Mol. Carcinog.* **36**, 130–141
59. Miura, Y., Kozuki, Y., and Yagasaki, K. (2003) *Biochem. Biophys. Res. Commun.* **305**, 160–165
60. Ramsby, M. L., Makowski, G. S., and Khairallah, E. A. (1994) *Electrophoresis* **15**, 265–277
61. De Palma, M., and Naldini, L. (2002) *Methods Enzymol.* **346**, 514–529