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Research Article

Hepatocyte growth factor differently influences Met-E-cadherin phosphorylation and downstream signaling pathway in two models of breast cells

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Abstract. E-cadherins are implicated in cell adhesion, and also in cell signaling by associating with tyrosine kinase-receptors such as Met, the hepatocyte growth factor (HGF) receptor. Using two different cellular models, *i.e.* MCF-7 (breast carcinoma) and MCF-10 (immortalized mammary) cells, we studied the possible mechanism(s) by which E-cadherins modulate the signaling pathways downstream of Met, leading to β-catenin-TCF transcriptional activity. In MCF-7, but not in MCF-10 cells, E-cadherins were remarkably associated with Met. Moreover, in MCF-7 cells both co-immunoprecipitation with anti-Met antibody and co-localization were increased by 30-min HGF treatment, which caused E-cadherin tyrosine phosphorylation. Also β-catenin in the co-immunoprecipitate was phosphorylated by HGF, probably favoring TCF activation. Consistently, after HGF treatment, β-catenin redistributed earlier in MCF-7 than in MCF-10 cells, with nuclear accumulation and activation of TOPFLASH gene reporter. Our results indicate a functional role of Met-E-cadherin interaction in MCF-7 cells through the amplification of the signaling downstream of HGF-Met triggering that involved c-Src and phosphoinositide-3-kinase activities.

Keywords. Met, hepatocyte growth factor, E-cadherins, β-catenins, breast carcinoma, transduction signal.

Introduction

Assembly of supramolecular complexes has been reported to occur at the membrane level to modulate and drive specific responses downstream of triggered growth factor receptors. The interaction with these receptors confers to adhesion molecules, although catalytically inactive, the ability to translate environmental cues into complex intracellular signals [1, 2].

E-cadherins are components of the adherens junctions, that might regulate endogenous β-catenin-TCF transcriptional activity [3]. Through the cytoplasmic domain, E-cadherins associate with β- and γ-catenins and then to the cytoskeleton. Dynamic and reversible modulation of E-cadherin expression occurs during ductal breast carcinoma progression, and E-cadherins are lost or down-regulated in the lobular tumor type [4]. Moreover, point mutation of the phosphorylation site on the cytosolic domain of E-cadherins or the tyrosine phosphorylation of β-catenins may cause the dissociation of the two molecules [5]. It is known that the multifunctional β-catenin-protein is implicated in adhesive complexes as well as in transcriptional co-activation. The free cytosolic form of β-catenin, which principally participates in the cell transduction signal leading to nuclear gene expression, might be influenced by E-cadherin interaction and the activation of receptor and non-receptor tyrosine kinases, such as c-Src [4, 5]. The existence of functionally distinct pools of cytosolic β-catenins suggests that there are specific mechanisms to regulate β-catenin signaling and to control its level of accumulation [6].

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Several studies have indicated an association between E-cadherins and tyrosine kinase receptors, suggesting that these molecules reciprocally regulate their activities to mount the final cellular response [7]. There are only few data concerning E-cadherin/Met association and the influence exerted on signal transduction in human normal and transformed cells. Met tyrosine kinase is the receptor for hepatocyte growth factor (HGF), and is present on cells of epithelial origin, such as breast and colon cells. Met activation evokes pleiotropic biological responses, often referred as 'invasive growth', which normally takes place during embryogenesis and tissue repair. Deregulation of this process is responsible for cancer progression and metastasis [8, 9]. Met and E-cadherins seem to localize at the periphery of colon and breast carcinoma cells [10]. In MDCK cells, Met and E-cadherins are also co-localized at cell adhesion sites and undergo co-endo-exocytosis after HGF treatment [11]. In MDCK cells transfected with HER2/ErbB2, a prognostic marker of breast cancer as Met, HGF/Met interaction converts the epithelial morphogenic program to cell invasion [12]. E-cadherins/Met association does not occur in normal mouse hepatocytes, and Met stimulated by HGF directly catalyzes the phosphorylation of β-catenins and their nuclear translocation [13]. However, the knowledge of the molecular events triggered by HGF and involved in the activation of β-catenin-TCF transcription factor in human breast cancer is very limited.

The present study focuses on mechanisms whereby E-cadherins may modulate the signaling pathway(s) downstream of Met activated by HGF and leading to β-catenin-TCF transcriptional activity. To point out the biomedical importance of this regulatory mechanism, we used two human cell models, MCF-7 (breast carcinoma) and MCF-10 (immortalized mammary epithelial) cells and examined Met-E-cadherin co-precipitation and co-localization, as well as tyrosine phosphorylation, in response to HGF. Early times after HGF stimulation were considered, when intracellular transduction signal(s) involving Met-E-cadherins-β-catenins was likely to be triggered. The presence in the supramolecular complex of β-catenins, their phosphorylation state and functional role in the two cell types were also evaluated. Thus, we studied the HGF-dependent intracellular redistribution of β-catenins, and the consequent activity of the gene reporter driven by β-catenins-TCF binding site multimer (TOPFLASH), as well as the role played by c-Src and phosphoinositide-3-kinase (PI3K) transducers.

Materials and methods

Materials. RPMI 1640, DMEM, Ham's F-10 and fetal bovine serum (FBS) were from Sigma Chemical Co. (St. Louis, MO). Recombinant human HGF was from R&D Systems (Abingdon, UK). Hybond ECL nitrocellulose membranes were from Amersham (Amersham Biosciences Europe GmbH, Italy). The polyclonal anti-Met (C12) and anti-vinculin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-E-cadherin (C20820) and anti-β-catenin (C19220) antibodies and the secondary antibodies FITC-conjugated goat anti-mouse (green) and CY3 goat anti-rabbit (red) were from Transduction Laboratories (Lexington, KY). Monoclonal anti-phosphotyrosine (pTyr) antibody (clone 4G10), anti-phospho-c-Src (Tyr-416) and anti-c-Src were from Upstate Biotechnology (Lake Placid, NY). Alexa Fluor 488 secondary antibody was from Molecular Probes (Eugene, OR). pRL-TK (Renilla luciferase reporter gene) was from Promega (Madison, WI). All other chemicals were of the highest grade available.

Cell cultures. Human breast carcinoma cells MCF-7 (European Cell Cultures Collection) were routinely maintained in RPMI 1640 medium containing 10% FBS. The human breast immortalized MCF-10 cells (European Institute of Oncology, Milan, Italy) were cultured in DMEM/F-10 supplemented with 10% FBS, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 20 ng/ml EGF, 0.1 µg/ml cholera toxin, 100 U/ml penicillin-streptomycin and 2 mM glutamine. The cells were starved (0.1% FBS) for 18–24 h. The starvation medium for MCF-10 cells was DMEM:Ham's (1 : 1) without EGF and cholera toxin but containing 2 mM glutamine. The cells were then treated with HGF (100 ng/ml), and used for total protein extraction. Preliminary experiments demonstrated that 100 and 200 ng/ml HGF gave the same effects.

Immunoprecipitation and Western blotting. Pools of 60×10^6 cells were lysed in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.25% deoxycholate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, leupeptin, and pepstatin A, and 1 mM Na_3VO_4 and NaF). For co-immunoprecipitation studies, samples corresponding to 1 mg and 100 µg protein were diluted with lysis buffer (final concentration 2.5 µg/µl), and were incubated overnight at 4 °C with 6.5 µg anti-Met antibody and 400 ng anti-E-cadherin antibody, respectively. Incubation was performed for 2 h at 4 °C in the presence of 80 µl (1 : 1) protein A-Sepharose bead slurry to allow coupling to the immune complex [14]. The immunoprecipitates were subjected to SDS-polyacrylamide 7% gel electrophoresis. The bands were transferred to the nitrocellulose filters, which were divided into two parts depending on the molecular weight of the proteins to analyze. The upper part of the filters was first probed with anti-pTyr antibody (3 µg/µl overnight at 4 °C), stripped and then reprobed with anti-E-cadherin antibody (1 : 2500 for 1 h at room temperature),

stripped and then reprobed with anti-Met antibody (1 : 200 overnight at 4 °C). The lower part of the filters was first probed with anti-pTyr antibody (3 μg/μl overnight at 4 °C), stripped and then reprobed with anti-β-catenin antibody (1 : 500 for 1 h at room temperature). The immunoblots were incubated for 1 h with the appropriate secondary antibodies coupled to horseradish peroxidase, followed by exposure to ECL plus chemiluminescence detection reagents and autoradiography. For each treatment, the bands corresponding to pTyr, Met, E-cadherin or β-catenin were quantified by densitometric analysis, and the relative fold-increases (E-Cad/Met, pTyr/Met, pTyr/E-Cad or pTyr/β-catenin) were calculated. Western blots were performed with 100 μg total cell lysate, and immunoblotting with anti-vinculin antibody was used to confirm equal loading.

Confocal scanning laser and fluorescence microscopy. MCF-7 cells (8×10^4) and MCF-10 cells (1×10^5) were seeded on sterile coverslips, previously placed in 24-multiwell plates, and were allowed to attach [15]. After starvation, the cells were treated with HGF (100 ng/ml) for 30 min, 1 h or 2 h and then were fixed with 4% paraformaldehyde solution. We observed that the specific fluorescent signals, evaluated by confocal and microscopy analyses, were not further increased by doubling the HGF concentration.

For the experiments with the confocal microscope, the cells permeabilized with 0.2% Triton X-100 were incubated at room temperature with anti-Met antibody (1 : 50) for 1 h, washed and then exposed to anti-E-cadherin antibody (1 : 5000) for 1 h. For secondary antibody immunoblotting, goat FITC-conjugated anti-mouse green (1 : 25) and goat CY3 anti-rabbit red (1 : 800) antibodies were used concomitantly. All the antibodies were diluted in 0.5% BSA. For negative control staining, only the secondary antibodies were incubated with the cells. No positive staining was observed with secondary antibodies alone. Nuclear staining was performed with DAPI (1 : 2000). The coverslips were mounted with Moviol and observed under Leica TCS SP2-A0BS confocal microscope. Images were taken at 260-nm intervals (63 × magnification) and analyzed with the appropriate software.

For fluorescence microscopy, the cells permeabilized with 0.2% Triton X-100 were incubated at room temperature with anti-β-catenin antibody (10 μg/ml) for 2 h followed by Alexa Fluor 488 antibody (1 : 800). Nuclear staining was performed with Hoechst 3342 (1 : 1000). The coverslips were mounted with Moviol and the cells were examined with a fluorescence microscope. Images were collected through the specimens at 40 × magnification and displayed on a computer screen [15].

Plasmids. TOPFLASH (TCF reporter plasmid) and FOPFLASH (mutant TCF binding sites) reporter constructs were generously given by Dr. B. M. Gumbiner (Memorial Sloan-Kettering Cancer Center, NY). The Src expression vectors for the wild-type (Srcwt) and mutated (Srcmut) forms were from Dr. S. Parson (University of Virginia, Charlottesville, VA). The SrcK295M (ΔSrc), the dominant negative form for Src, was from Dr. W.C. Horne (Yale University, CT). The SRα(XbaI)Δp85 (Δp85), the dominant negative form of PI3K-p85 subunit, was from Dr. P. Raynal (INSERM, Toulouse, France).

Transient transfection assay. MCF-7 and MCF-10 cells, seeded in 24-multiwell plates, were allowed to reach 70–80% confluence (2×10^5 cells) and then were transfected using Fugene-6 (Roche) with the gene reporters TOPFLASH, FOPFLASH, Src wt or Src mut (200 ng/well) and/or with the dominant negative ΔSrc or Δp85 (1 μg/well) [15, 16]. To evaluate the luciferase activity, pRL-TK was co-transfected for normalization. After 8 h, all the cells underwent overnight starvation before HGF treatment (200 ng/ml). The cells were harvested after 24 h, and Firefly and Renilla luciferase activities were measured with a dual luciferase assay system (Promega). Some cells transfected with c-Src wt, starved and treated with HGF (200 ng/ml) for 1 h, were used for total protein extraction together with untransfected cells, exposed or not to HGF. Western blot analyses were performed with 20 and 100 μg protein for transfected and untransfected cells, respectively. Immunoblots with anti-phospho-Src (Tyr-416) and anti-c-Src were performed.

Statistical analysis. Densitometric values were analyzed by analysis of variance, with $p < 0.05$ considered significant. Differences from controls were evaluated on original experimental data, and we then assigned the arbitrary value of 1 to controls.

Results

HGF enhanced Met-E-cadherin association and their tyrosine phosphorylation in MCF-7 but not in MCF-10 cells. To evaluate the possible co-immunoprecipitation and co-localization of Met and E-cadherins as well as the tyrosine phosphorylation state under different experimental conditions, we used MCF-10, an immortalized mammary epithelial cell line, and MCF-7 breast carcinoma cells (Fig. 1). The cells were exposed to HGF for various times or were cultured at different density conditions.

Total protein extracts, prepared from 30- and 60-min HGF-treated cells, were immunoprecipitated with anti-Met antibody and were immunoblotted as shown in Figure 1a. After starvation, twofold more Met was immunoprecipitated in MCF-10 than in MCF-7 cells while threefold more E-cadherin co-immunoprecipitated (E-Cad/Met) in MCF-7 than in MCF-10 cells. E-Cad/Met

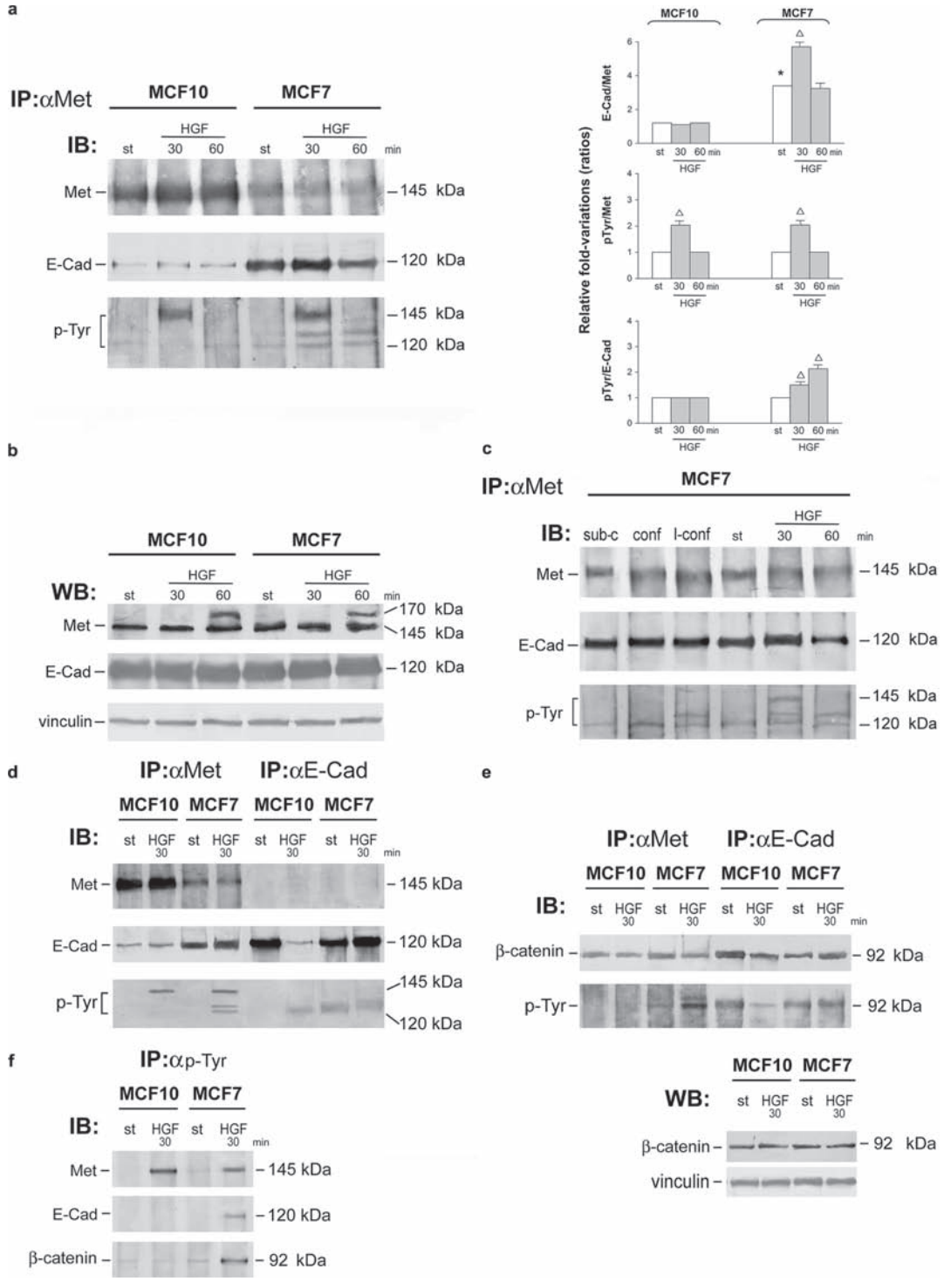

Figure 1. Met association with E-cadherins in MCF-7 and MCF-10 HGF-treated cells. (*a*) Met protein was immunoprecipitated, and immunoblots were performed. Data from the densitometric analysis are shown as relative fold variations of the ratios: E-Cad/Met, pTyr/Met and pTyr/E-Cad. Each value is the mean ± SE of experiments performed in triplicate. *$p < 0.05$ for starved (st) MCF-7 cells vs st MCF-10 cells; $^{\Delta}p < 0.05$ for HGF-treated cells vs corresponding st cells. (*b*) Western blot analysis of total protein extracts after HGF treatment. Immunoblot with anti-vinculin antibody was used for normalization. (*c*) Met protein was immunoprecipitated using total protein extracts prepared from subconfluent (50% confluence, subc), confluent (conf), long-confluent (72 h confluence, l-conf) and HGF-treated MCF-7 cells, and immunoblots were performed. The experiments were repeated three times with similar results. (*d–f*) After HGF treatment, immunoprecipitations with anti-Met (IP:αMet), anti-E-cadherin (IP:αE-Cad) and anti-pTyr (IP:αp-Tyr) antibodies were carried out, and immunoblots were performed. Western blot analysis of total protein extracts after HGF treatment and immunoblotting with anti-β-catenin antibody were performed. Immunoblot with anti-vinculin antibody was used for normalization. The experiments were repeated three times with similar results.

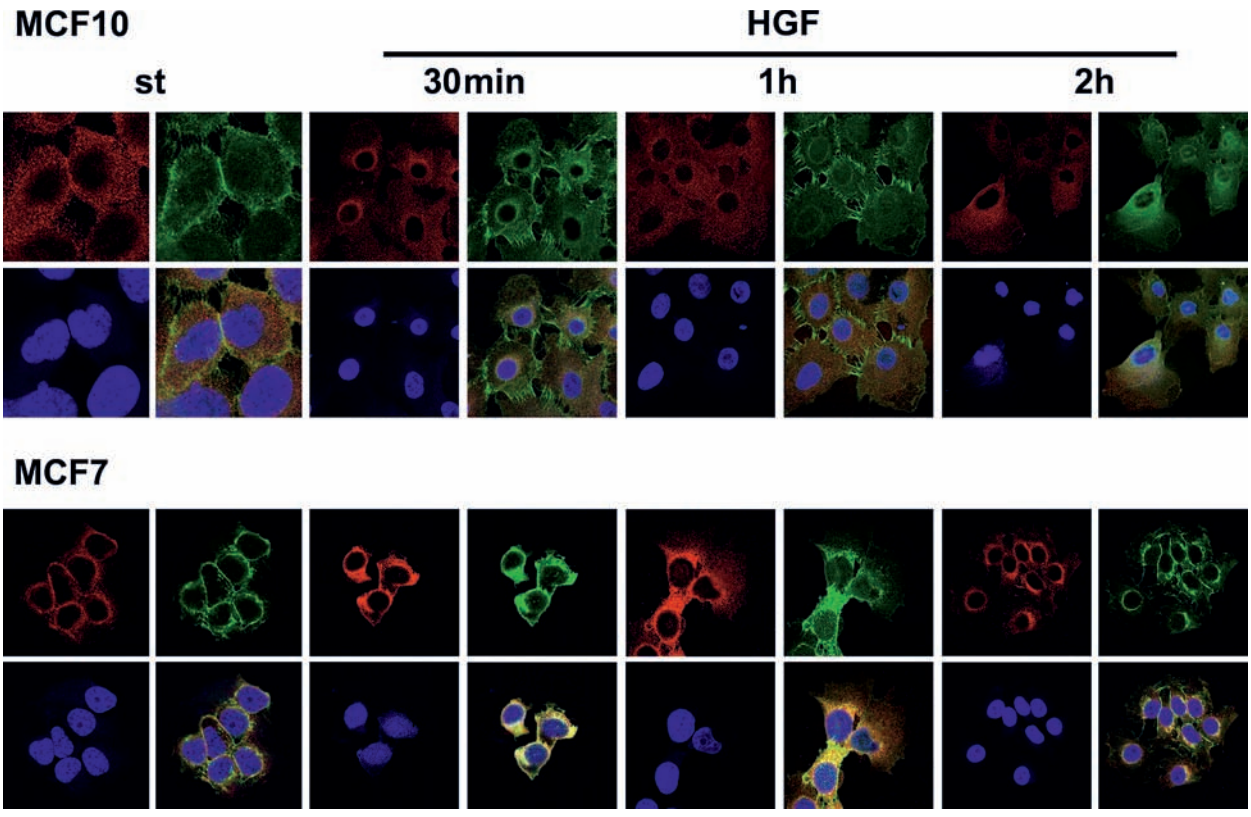

Figure 2. Co-localization of Met and E-cadherins seen in HGF-treated MCF-7 cells was absent in MCF-10 cells. The cells were plated in multiwells, treated with HGF for various times, and then fixed with 4% paraformaldehyde. After permeabilization, the cells were incubated with anti-Met antibody (red, after reaction with the secondary antibody) followed by anti-E-cadherin antibody (green, after reaction with the secondary antibody), and then were stained with DAPI (blue). The fourth panel is the merged image (yellow). Confocal laser scanner analysis was performed (magnification 63 ×). Zoom shows the starved (st) MCF10 cells.

doubled in 30-min HGF-treated MCF-7 cells, decreasing thereafter to the starvation value. Met was tyrosine phosphorylated (pTyr/Met) in MCF-10 and MCF-7 cells at 30 min after HGF treatment, while E-cadherin was tyrosine phosphorylated (pTyr/E-Cad) in MCF-7 cells at 30 and 60 min after HGF (about twofold increase of the120-kDa band). The higher molecular mass band phosphorylated in tyrosine might correspond to an E-cadherin-associated protein in HGF-treated MCF-7 cells. Consistently, in the immunoprecipitate performed with anti-pTyr antibody, we observed only the 120-kDa band by immunoblotting with anti-E-cadherin (Fig. 1f).

Western blot analysis showed that the steady-state levels of mature (145 kDa) Met subunit were similar in the two cell lines used and were unchanged after HGF treatment, while the level of the receptor precursor (170 kDa) was increased by 60-min HGF exposure in both the cell lines. E-cadherin protein levels were the same in treated and untreated cells (Fig. 1b).

To evaluate the effect of cell density on Met and E-cadherin co-immunoprecipitation, we also performed experiments with subconfluent (50%), confluent and long-confluent (72 h) MCF-7 cells using anti-Met antibody (Fig. 1c). Only long-confluence increased tyrosine phosphorylation of E-cadherins (pTyr/E-Cad), as shown after HGF treatment. The levels of co-immunoprecipitated E-cadherin (E-Cad/Met) was unaffected by cell density.

When immunoprecipitation was performed with anti-E-cadherin antibody, Met did not seem to co-immunoprecipitate either in MCF-10 or in MCF-7 cells (Fig. 1d). We used samples corresponding to 1 mg and 100 µg protein for the immunoprecipitates with anti-Met and anti-E-cadherin antibodies, respectively, which permitted the concomitant chemiluminescence reaction and X-ray exposure of the filters. The same results were obtained with increasing quantities of proteins up to 1 mg (data not shown). However, E-cadherin was immunoprecipitated with both anti-Met and anti-E-cadherin antibodies under starvation conditions, although to a very different extent in the two cell lines used. In MCF-10 cells, considering immunoprecipitates with anti-E-cadherin, HGF treatment remarkably reduced the level of E-cadherin that was tyrosine phosphorylated. In MCF-7 cells, E-cadherin was strongly immunoprecipitated and phosphorylated after starvation and HGF treatment.

Figure 1e shows that β-catenins were associated with Met and E-cadherins in starved MCF-10 and MCF-7 cells. HGF treatment (30 min) of MCF-7 cells increased tyro-

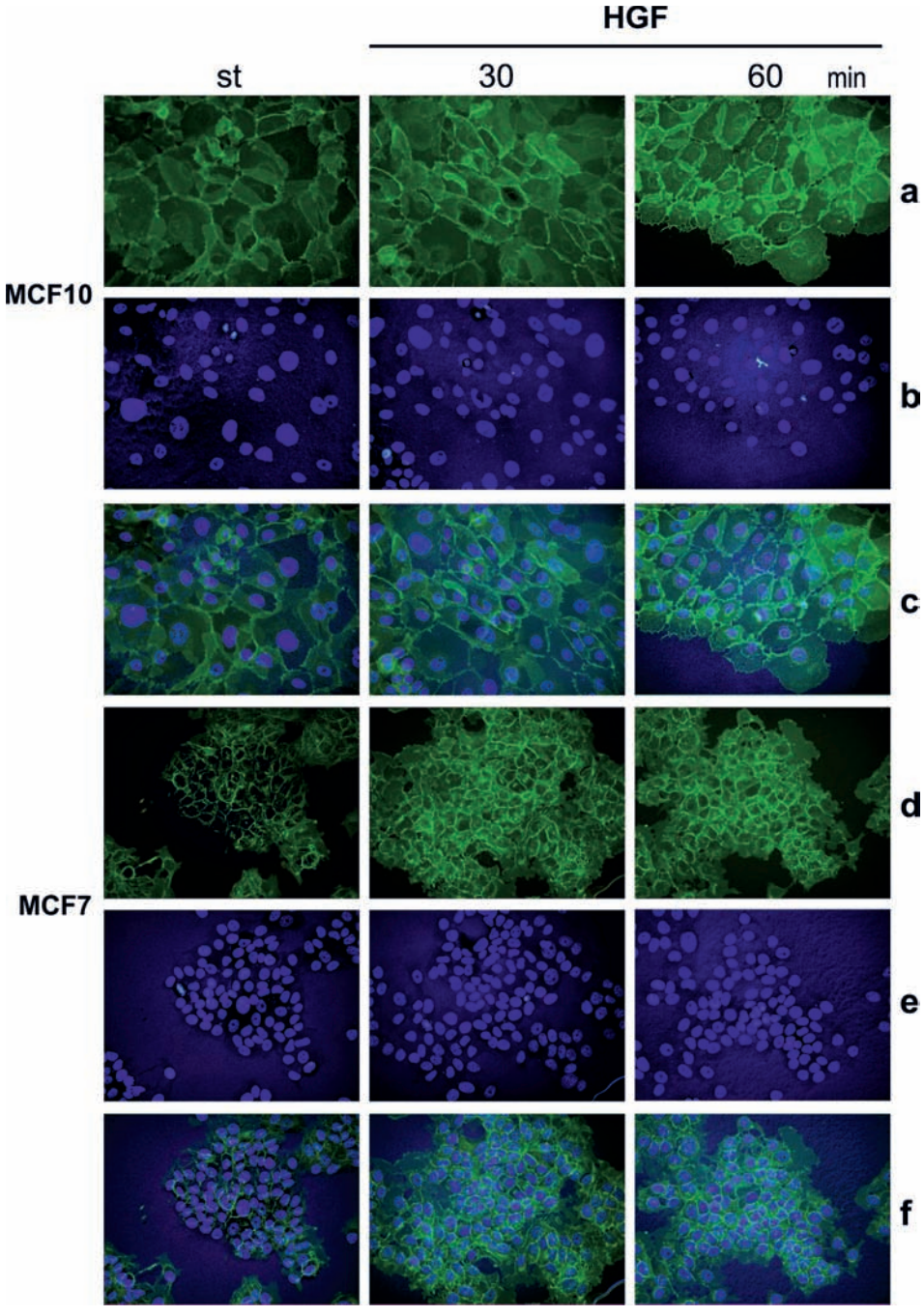

Figure 3. *β*-catenin protein expression and redistribution in HGF-treated MCF-10 and MCF-7 cells. The cells were seeded on coverslips, treated with HGF for 30 or 60 min, and then fixed with 4% paraformaldehyde. After permeabilization, incubation was performed with anti-*β*-catenin antibody and then with the appropriate secondary antibody (green, *a*, *d*). The nuclei were stained with DAPI (blue, *b*, *e*). *c*, *f* Merged image. Images were taken using fluorescence microscopy at 40 × magnification.

sine phosphorylation of *β*-catenins co-immunoprecipitated with Met because the 92-kDa band was enhanced and a faint higher molecular mass band appeared [17]. In the immunoprecipitates with anti-E-cadherin antibody and MCF-10 protein extracts, *β*-catenins seemed to be strongly associated with the small quantities of E-cadherins remaining at 30 min after HGF treatment and were slightly phosphorylated. Using MCF-7 cell extracts, *β*-catenin phosphorylation was elevated under starvation and was unaffected by HGF. Western blot analysis showed that the steady-state levels of *β*-catenin were similar in the two cell lines under the different treatments (Fig. 1e). A confirmation of these results was given by immunoprecipitation experiments with anti-pTyr antibody (Fig. 1f). After HGF treatment, we observed that Met was present in the immunoprecipitates performed with MCF-10

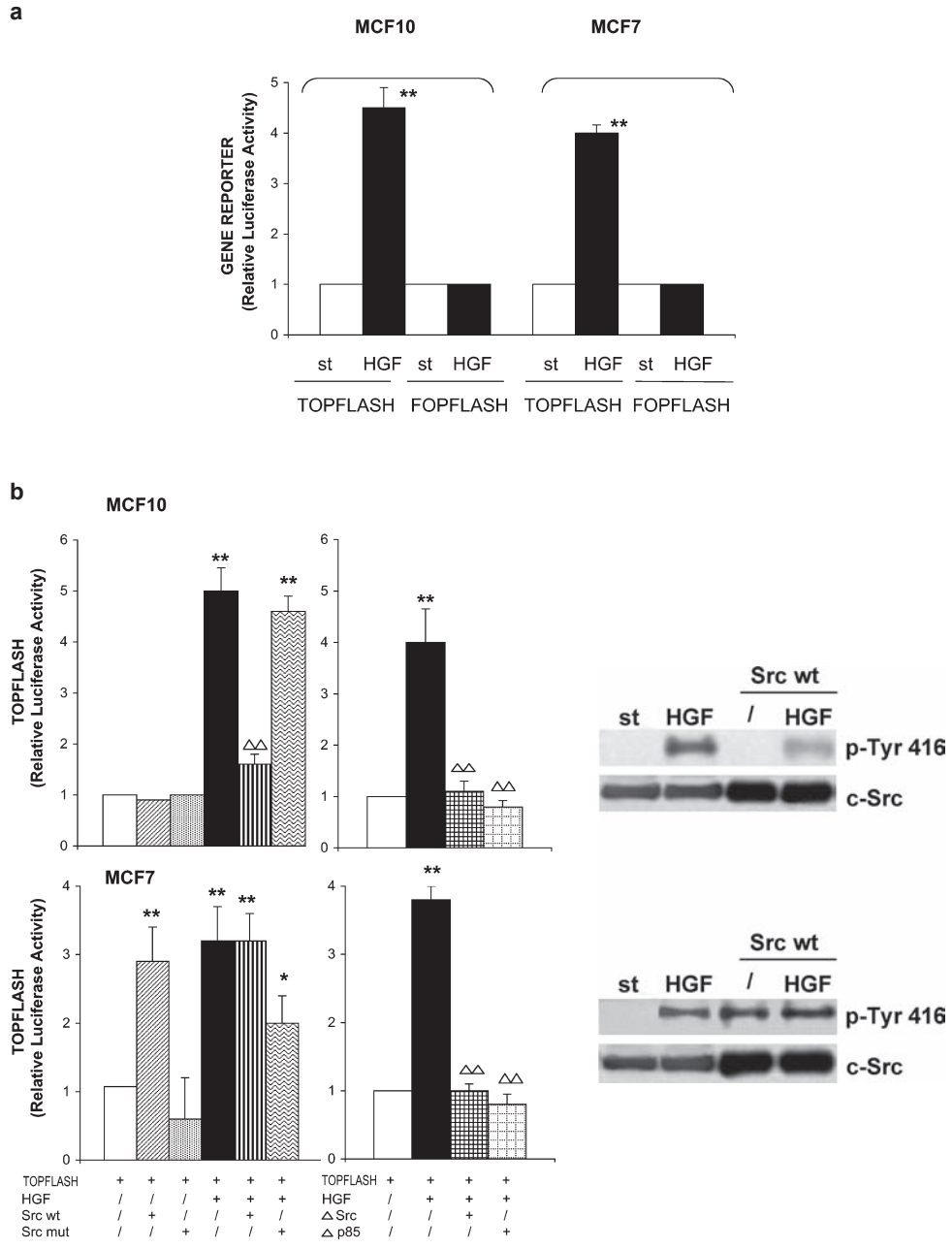

Figure 4. Luciferase activity of TOPFLASH and FOPFLASH after HGF treatment of MCF-10 and MCF-7 cells. (*a*) The cells, transiently transfected with TOPFLASH or FOPFLASH gene reporters, were starved and then treated with HGF. The numbers indicate the fold changes of luciferase activity vs starved transfected cells. The data are the means ± SE of three independent experiments performed in duplicate. **$p < 0.005$ vs st cells. (*b*) The cells, transiently co-transfected with TOPFLASH gene reporter and the expression vector for Src wild-type (wt) or Src mutated (mut) or for the dominant negative ΔSrc or Δp85, were starved and then treated with HGF. The numbers indicate the fold changes of luciferase activity vs starved transfected cells. The data are the means ± SE of three independent experiments performed in duplicate. **$p < 0.005$ vs st cells; $^{\Delta\Delta}p < 0.005$ vs HGF-treated cells. Inset: Western blot analyses of protein extracts from Src wt transfected and untransfected cells, treated or not with HGF. Immunoblots were performed with anti-c-Src and anti-phospho-Src (Tyr-416) antibodies. Representative autoradiograms of three independent experiments.

and MCF-7 cell protein extracts, while E-cadherins and *β*-catenins were co-immunoprecipitated only in MCF-7 cells.

Figure 2 shows the confocal scanner laser images of MCF-10 and MCF-7 cells exposed to HGF for various times. In starved MCF-10 cells, Met (red) seemed to be localized in the plasma membrane and also intracellularly [18, 19], while E-cadherins (green) were principally localized in the membrane at the cell-cell adhesion sites. After 30 min and 1 h of HGF treatment, E-cadherins were notably present in destabilized junctional complexes [20, 21] and appeared diffusely also inside the cell. After 2 h

of HGF treatment, the E-cadherins were mostly localized around the nucleus, while Met seemed to be at the leading-edge extension. In MCF-7 cells we observed a certain co-localization of Met and E-cadherins at the cell membrane level where the cells established contacts [11]. The dimensions of the MCF-7 cells were about a quarter those of the MCF-10 cells, and the cytosol was really scarce in starved cells forming colonies. After 30-min HGF treatment, Met and E-cadherins were strongly associated, as shown in the merge image (yellow), and seemed to accumulate asymmetrically [19]. After 1-h HGF treatment, co-localization was observed only in the central part of the image, where the cells were still in contact. Also in this case, we observed Met (red) principally at the leading-edge extension. After 2-h HGF treatment, the two molecules appeared to be completely internalized inside the cell around the nucleus.

HGF regulated β-catenin-pathway in MCF-10 and MCF-7 cells. Immunofluorescence experiments were performed to evaluate the possible effect of HGF on the redistribution of β-catenins in the two cell lines used (Fig. 3). We observed that β-catenins were localized to the cell membrane in starved MCF-10 and MCF-7 cells. After HGF treatment, both the cell types stained strongly for β-catenins in a predominantly nuclear and cytoplasmic pattern, in addition to membranous staining. It is worth noting that in carcinoma cells MCF-7 an extensive redistribution between cytosol and nucleus occurred after 30 min of HGF treatment, and in normal epithelial cell model MCF-10 principally after 60 min. The merge image gave a better idea of nuclear localization of β-catenins after HGF treatment, which occurred earlier in MCF-7 than in MCF-10 cells.

We studied the consequences of β-catenin redistribution by analyzing the functional activity of β-catenin-mediated transactivation using the TOPFLASH and FOPFLASH gene reporter plasmids (Fig. 4a). TOPFLASH contains multiple TCF/LEF consensus sites upstream of a c-fos minimal promoter driving luciferase expression. The binding of β-catenin to TCF and its translocation to the nucleus induces luciferase activity. FOPFLASH is a construct lacking functional TCF/LEF consensus sites, and is therefore β-catenin independent [22]. HGF enhanced the TOPFLASH activity (4.5–4-fold) in MCF-10 and MCF-7 cell lines.

As shown in Figure 4b, we co-transfected MCF-10 and MCF-7 cells with TOPFLASH and the expression vectors for c-Src wild-type (Src wt) and mutated (Src mut) in the presence or the absence of HGF to evaluate whether c-Src tyrosine kinase was involved in the β-catenin-TCF activity, and whether or not this signal-transduction pathway was common to that triggered by HGF, leading to β-catenin-TCF activation. The mutation present in the kinase-defective c-Src is a valine substitution for alanine at residue 430 in the catalytic domain [23]. To extend the study to the transduction pathways downstream of HGF/Met responsible for β-catenin-TCF activity, the two cell types were also co-transfected with TOPFLASH and the dominant negatives for PI3K (Δp85) or for c-Src (ΔSrc). Δp85 is a mutant form of the PI3K regulatory subunit p85, which cannot interact with the catalytic subunit p110, thus inhibiting the enzyme activity [24]. ΔSrc is a kinase dead-Src with a single amino acid mutation at residue 295 [25]. In MCF-10 cells, Src wt and Src mut did not affect the basal TOPFLASH activity, while Src wt prevented HGF-dependent stimulation of luciferase activity of the gene reporter. In MCF-10 cells co-transfected with Src mut, the stimulatory effect of HGF on TOPFLASH activity was unmodified. In MCF-7 cells, HGF increased TOPFLASH activity similarly to Src wt expression vector, but the two effects were not additive. The expression vector for Src mut slightly, but not significantly, reduced TOPFLASH activity in the presence of HGF, while it did not modify the basal activity. We also performed Western blots in the Src wt-transfected cells, treated or untreated with HGF. For comparison, endogenous levels of c-Src in untransfected cells are shown (Fig. 4, *inset*). c-Src expressed by construct transfection was phosphorylated at tyrosine 416 only in MCF-7 cells, in the presence or the absence of HGF treatment, indicating a possible activation of the protein kinase in these cells that was independent of HGF. The endogenous protein in the two cell lines was highly phosphorylated after HGF treatment. In starved cells, c-Src phosphorylation was very low and required longer times of exposure to be appreciated. Immunoblots with anti-vinculin antibody were performed for normalization (data not shown). Figure 4b also shows that the co-transfection of ΔSrc or Δp85 and TOPFLASH completely prevented the gene reporter activation after HGF in both cell lines. The dominant negative expression vectors did not affect the basal TOPFLASH activity (data not shown).

Discussion

The growth-suppressive activity of E-cadherin seems to be adhesion independent and to result from inhibition of the β-catenin/TCF signaling pathway [6]. Here we studied whether HGF/Met coupling activated the transduction pathway leading to β-catenin/TCF activation, and the possible role played by E-cadherins in tumor breast cancer cells, MCF-7, and in mammary epithelial model MCF-10.

In MCF-7 breast carcinoma cells, a remarkable association between Met and E-cadherin was observed, which seemed to be specific for transformed cells. In MCF-10 immortalized breast epithelial cells, E-cadherin levels were very low in the immunoprecipitates formed

with anti-Met antibody. Consistently, HGF increased tyrosine phosphorylation of Met in both cell types, but only E-cadherins seemed to be tyrosine phosphorylated in MCF-7 cells. Moreover, in MCF-7 cells, Met and E-cadherin co-localized at cell-cell adhesion sites [11], and also accumulated asymmetrically within 30 min of HGF treatment as shown with the confocal microscope. Met, predominantly located at the plasma membrane in unstimulated cells, was rapidly internalized following HGF treatment at early endosomes; at later time points, the localization pattern was more perinuclear. The perinuclear compartment includes the late endosomes/lysosomes, *i.e.* the major route for Met degradation after proteasome delivery, as well as the Golgi, corresponding to a recycling compartment or containing newly synthesized Met (p170) [26]. Experiments are underway to clarify whether Met/E-cadherin interaction in internal compartments of MCF-7 cells is important for the activation of β-catenin/TCF signaling and nuclear gene expression, as reported for Met in other experimental conditions [18, 27].

E-cadherin phosphorylation seemed to play a fundamental role in Met-E-cadherin functional interaction in MCF-7 tumor cells, through the amplification of the signaling downstream of Met triggered by HGF. Met tyrosine kinase or more likely c-Src tyrosine kinase might be involved in E-cadherin phosphorylation. In many cases, cell stimulation with growth factors causes activation of c-Src [28, 29], which may phosphorylate proteins of adherens junctions such as E-cadherins [30–32].

The tyrosine phosphorylation of E-cadherins might mediate the direct interaction with β-catenins and/or with Shc and PI3K [7, 31, 33, 34]. β-catenins play critical roles in morphogenesis and tumor growth, through the dual function in adhesive complexes and as a tranducer/transcriptional regulator in numerous signaling pathways [6]. β-catenins were more associated with Met in MCF-7 tumor cells than in normal epithelial MCF-10, and, after HGF treatment, a decrease (–50%) in the association occurred in MCF-7 cells consistent with previous data [13]. In addition, β-catenins co-immunoprecipitated with E-cadherins under our experimental conditions, and a stabilization of these β-catenins after HGF treatment was observed in MCF-7, but not in MCF-10 cells. The β-catenins present in the E-cadherin complex in MCF-7 cells were concomitantly tyrosine phosphorylated, and therefore might be less polyubiquitinated. In the case of β-catenins, tyrosine phosphorylation causes conformational changes that decrease its affinity for axin, a step required for ubiquitination and proteasomal degradation of β-catenins [30].

E-cadherins may be able to sequester the transcriptionally competent pool of β-catenin [6]. In MCF-7 carcinoma cells, the presence in the Met complex of E-cadherins as well as of β-catenins did not restrain, but probably facilitated, β-catenin phosphorylation by Met tyrosine kinase-signaling pathway, followed by nuclear translocation, as compared with HGF-treated MCF-10 cells. Consistently, in MCF-7 cells, HGF caused a more rapid redistribution of β-catenins from cell membrane to cytosol and nucleus in immunofluorescence experiments. Thus, HGF, a cytokine of the tumor microenvironment, determined a cell pattern of β-catenins similar to that observed in tumor specimens [35, 36]. In MCF-10 cells treated with HGF, β-catenins were also notably localized in 'moving structures', as reported for colon carcinoma cells cultured on lamina [36].

In MCF-10 and MCF-7 cells exposed to HGF, the nuclear translocation of β-catenins seemed to activate the TCF transcription factor, as demonstrated by the increase in TOPFLASH luciferase activity after HGF treatment. These data are in agreement with HGF regulation of GSK3β activity and β-catenin signaling in mouse mammary epithelial cells [37]. The mechanisms and the fate of β-catenins upon tyrosine phosphorylation in response to certain growth stimuli is still largely unknown. Tyrosine phosphorylation may favor β-catenin stabilization, contrary to serine phosphorylation catalyzed by G3SK, which enhances β-catenin degradation [30].

The activation of Met tyrosine kinase after HGF binding mediates the interaction of signaling molecules, such as Src, PI3K, PLC, and Grb2, with the multifunctional docking site. Transduction pathways activated downstream, leading to functional alterations of the cells, such as growth and motility, may participate in β-catenin stabilization and the consequent TCF activation [9, 15, 28, 29]. We, therefore, studied PI3K and c-Src involvement in the TOPFLASH gene reporter activity. Our data show that c-Src was along a common signaling pathway triggered by HGF/Met in MCF-7 cells because the effect of HGF stimulus and c-Src protein expression were similar but not additive. c-Src expressed in MCF-7 cells seemed to be active independently of HGF treatment. Moreover, PI3K or Src blockade completely prevented TOPFLASH activity increase in response to HGF. In conclusion, in MCF-7 breast tumor cells, β-catenin was the substrate of HGF-stimulated Met tyrosine kinase or of a tyrosine kinase activated downstream. A suggested mechanism might be the activation of Src by direct interaction with Met and, in the cascade, the phosphorylation of p85, the regulatory subunit of PI3K. Consistently, c-Src is known to influence p85 interaction with β-catenin, which causes stabilization of the cytosolic β-catenin preventing the binding to a 'destruction complex' [28]. Additionally or alternatively, PI3K may inhibit the catalytic activity of GSK-3β that participates in the control of β-catenin proteasomal-degradation. The β-catenin-PI3K interaction seems also to favor PI3K translocation from the cytosol to the nucleus, where the lipid kinase probably activates the β-catenin-mediated transcription [33].

Recently, our knowledge on the role of PI3K in the regulation of signal transduction, cytoskeletal rearrangements

and membrane trafficking has expanded considerably [38]. Evidence is emerging that members of the PI3K superfamily and components of PI3K signaling play a role in the development of many human cancers. This complex pathway is known to be involved in the regulation of cell growth, differentiation, motility, proliferation, and survival, and hence the PI3K pathway components have become potential targets for the control of the growth and spread of cancers [34]. PI3K may be induced by HGF, a stimulus of the tumor microenvironment, and is implicated in TCF activation probably through β-catenin stabilization. Thus, the blockade of HGF/Met interaction or the PI3K signaling downstream may be a method to inhibit a pathway involved in neoplastic cell proliferation.

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