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Src and FAK Mediate Cell–Matrix Adhesion-Dependent Activation of Met During Transformation of Breast Epithelial Cells

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ABSTRACT

Cell–matrix adhesion has been shown to promote activation of the hepatocyte growth factor receptor, Met, in a ligand-independent manner. This process has been linked to transformation and tumorigenesis in a variety of cancer types. In the present report, we describe a key role of integrin signaling via the Src/FAK axis in the activation of Met in breast epithelial and carcinoma cells. Expression of an activated Src mutant in non-neoplastic breast epithelial cells or in carcinoma cells was found to increase phosphorylation of Met at regulatory tyrosines in the auto-activation loop domain, correlating with increased cell spreading and filopodia extensions. Furthermore, phosphorylated Met is complexed with β1 integrins and is co-localized with vinculin and FAK at focal adhesions in epithelial cells expressing activated Src. Conversely, genetic or pharmacological inhibition of Src abrogates constitutive Met phosphorylation in carcinoma cells or epithelial cells expressing activated Src, and inhibits filopodia formation. Interestingly, Src-dependent phosphorylation of Met requires cell–matrix adhesion, as well as actin stress fiber assembly. Phosphorylation of FAK by Src is also required for Src-induced Met phosphorylation, emphasizing the importance of the Src/FAK signaling pathway. However, stimulation of Met phosphorylation by addition of exogenous HGF in epithelial cells is refractory to inhibition of Src family kinases, indicating that HGF-dependent and Src/integrin-dependent Met activation occur via distinct mechanisms. Together these findings demonstrate a novel mechanism by which the Src/FAK axis links signals from the integrin adhesion complex to promote Met activation in breast epithelial cells. J. Cell. Biochem. 107: 1168–1181, 2009.

KEY WORDS: SRC; FAK; MET; CELL ADHESION; TRANSFORMATION; BREAST CANCER

The receptor tyrosine kinase (RTK) Met is frequently over-expressed in many types of human invasive carcinomas including breast [Tuck et al., 1996; Ghoussoub et al., 1998; Ocal et al., 2003], lung [Tsao et al., 1998] and ovarian [Huntsman et al., 1999] cancers, and this high level of expression correlates with poor patient survival. There is also evidence that integrin-based cell adhesion is required for neoplastic transformation of breast epithelial cells by activated oncogenes such as Her2/neu [White et al., 2004; Guo et al., 2006], and blocking β1 integrins causes reversal of the transformed phenotype of human breast carcinoma cells in 3-D culture [Weaver et al., 1997]. Moreover, cell–matrix adhesion has been shown to promote ligand-independent activation of Met and is linked to transformation and tumorigenesis by Met in a variety of cancers [Rusciano et al., 1995, 1998, 1999; Wang et al., 1996, 2001]. Thus co-operative integrin and Met signaling may be one important mechanism contributing to aberrantly high Met activation levels in invasive breast cancer.

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The mechanisms linking integrin and Met signaling are not fully understood. A possible candidate is the non-receptor tyrosine kinase Src, which is hyperactivated in many cancer types [Ottenhoff-Kalff et al., 1992, 1995; Verbeek et al., 1996], and is required for polyoma middle T-induced mammary tumorigenesis in transgenic mice [Guy et al., 1994]. Src is a key player in promoting disruption of cadherin junctions in carcinoma cells, and this process requires integrin-dependent adhesion [Avizienyte et al., 2002]. Src becomes activated by integrin- and RTK-mediated signaling, translocates to focal adhesions, and complexes with a variety of substrates including p130cas [Nakamoto et al., 1996] and FAK [Thomas and Brugge, 1997; Lin et al., 2004]. In a model proposed by Moro et al. [2002] a macromolecular complex, comprised of the intracellular domain of β1 integrin, Src, p130Cas and EGF receptor, was shown to modulate EGF receptor activity. FAK has also been shown to be critical for ErbB2/ErbB3 receptor activation in oncogenic transformation [Benlimame et al., 2005], and interaction of FAK with Met is required for HGF-induced cell invasion [Chen and Chen, 2006]. Furthermore, the activity of other RTKs including insulin-like growth factor receptor-I [Peterson et al., 1996], platelet-derived growth factor-β receptor [Hansen et al., 1996], and the Met-related Ron receptor [Danilkovitch-Miagkova et al., 2000] are regulated by Src and integrins. Recent studies also support a role of Src in regulating integrin cross-talk with Met in transformed cells [Sridhar and Miranti, 2006; Yamamoto et al., 2006], however the mechanism and cell adhesion conditions by which Src regulates Met activation are not clearly known.

We have previously shown that association of Src with its substrate FAK and phosphorylation of tyrosine 577 of FAK requires cell matrix adhesion [Lin et al., 2004]. We therefore investigated the role of Src and FAK in cell–matrix adhesion-dependent activation of Met. Our results show that an activated Src mutant enhances Met activation in non-neoplastic breast epithelial cells which do not express HGF. We further showed that genetic or pharmacological inhibition of Src family kinases reduces constitutive Met tyrosine phosphorylation in breast carcinoma cells expressing HGF and high endogenous Src activity. Interestingly, Src-dependent Met activation in both epithelial and carcinoma cells is tightly linked to cell–matrix adhesion, consistent with an HGF-independent mechanism. Tyrosine phosphorylation of FAK by Src is also required for this process. In contrast, stimulation of Met with exogenous HGF is refractory to Src inhibition in epithelial cells. Overall, our findings imply a regulatory role for the Src/FAK axis in integrin-dependent Met activation, which is distinct from HGF-dependent pathways of Met activation during mammary tumorigenesis.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

Rat anti-mouse monoclonal antibodies specific for the external domains of α5 (BMA5-1) and β1 (MB1-2) integrins and rabbit polyclonal antibodies specific for anti-β1 and anti-α5 integrin cytoplasmic domains were prepared as described previously [Fehlner-Gardiner et al., 1999]. Mouse monoclonal phospho-tyrosine [pY99] and mouse monoclonal anti-Met (B-2) antibodies were from Santa Cruz Biotechnology, Inc. (San Diego, CA). Rabbit polyclonal anti-Met[pY1230,1234,1235] and rabbit polyclonal Src[pY418] antibodies were kindly provided by Medicore (Montreal, QU). Mouse anti-phospho-tyrosine [PY20] antibody was from BD Transduction Laboratories (Mississauga, ON). Mouse monoclonal anti-v-Src (Ab-1) antibody was obtained from Oncogene Research Products (La Jolla, CA). Mouse monoclonal anti-Myc antibody was from Sigma–Aldrich (Oakville, ON). Sheep anti-mouse and donkey anti-rabbit immunoglobulin G horseradish peroxidase antibodies were from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Protein A sepharose and anti-rat IgG coated agarose beads were obtained from Sigma (Oakville, ON). Reconstituted bovine plasma fibronectin was from Invitrogen Life Technologies (Burlington, ON). Recombinant hepatocyte growth factor (HGF) was a gift from Dr. R. Schwall (Genentech, Inc., San Francisco, CA). Src family kinase inhibitors PD180970 and Dasatinib were obtained from Pfizer Global Research and Development (Ann Arbor, MI) and Sigma–Aldrich, respectively. Recombinant HGF was obtained from Invitrogen Life Technologies.

CELL LINES AND TISSUE CULTURE

The SP1 cell line was previously established from a spontaneous non-metastatic murine mammary intraductal adenocarcinoma from a retired CBA/J female mouse of 18 months, from Jackson Laboratory, and is a heterogeneous cell line [Carlow et al., 1985; Elliott et al., 1992], which co-expresses HGF and phosphorylated Met [Rahimi et al., 1998]. SP1 cells are cultured in complete Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Life Technologies), containing 10% FBS, 1% L-glutamine-200 mM and 1× antibiotic/antimycotic (Invitrogen Life Technologies). HC11 is a mammary epithelial cell line [Doppler et al., 1989], which expresses Met but not HGF [Wojcik et al., 2006]. HC11 cells are cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 2 mM L-glutamine, 10% FBS, 5 μg/ml insulin, and 10 ng/ml EGF. FAK+/+ and FAK−/− embryonic fibroblasts were kindly provided by Dr. D. Ilic (University of California). SYF−/− and SYF+/+ embryonic fibroblasts, and SYF−/− cells re-expressing Src (SYF−/− + Src) were obtained from ATCC (clones CRL-2459, CRL-2497, CRL-2498 respectively) [Klinghoffer et al., 1999]. All fibroblast lines were originally transformed with simian virus 40 large T antigen, and were cultured in complete DMEM medium. All cell lines used tested negative in a PCR-based mycoplasma assay (Sigma–Aldrich).

CELL TRANSFECTION

SP1 cells were stably transfected with an activated chicken Src mutant (SrcY527F) or kinase dead Src mutant (K295R, Y527F) in a pBabe(puro) expression vector, previously made in this laboratory [Hung and Elliott, 2001]. All transfections were carried out with Lipofectamine Plus reagent (Invitrogen Life Technologies). Stable transfections were selected with puromycin (4 μg/ml, Sigma–Aldrich), and individual clones were isolated. In some experiments, HC11 cells expressing SrcY527F were transduced with wild-type FAK or a dominant negative FAK(407-925) mutant (Y407F, Y576F, Y577F, Y861F, and Y925F) in a PWZL(hygro) vector and pooled lines were selected with hygromycin B (Sigma–Aldrich; 800 μg/ml) [Avizienyte et al., 2002]. Exogenous protein expression in each

clone was confirmed using indirect immunofluorescence and western blotting.

CELL CULTURES AND TREATMENT CONDITIONS

For serum starvation, cells were washed three times with pre-warmed (37°C) serum-free DMEM medium. After the final wash, cells were incubated overnight in serum-free DMEM (8 ml). For exogenous HGF treatment, 20 ng/ml (cell migration assays) and 40 ng/ml (adhesion assays) of recombinant HGF in 5 ml of DMEM with 0.5 mg/ml bovine serum albumin (BSA) (Fisher Scientific) were added. Control plates received the same medium without HGF. For non-adherent conditions, cultured cells were washed with PBS, then incubated with 0.5 mM EDTA in PBS to loosen cells from the plate. Cells were then harvested using DMEM with 0.5 mg/ml BSA (10 ml per culture equivalent), and were kept in suspension by inversion of the tube every 10 min. Cells were incubated at 5% CO_2 and 37°C for the times indicated.

For coating plates with cell–matrix substratum, fibronectin (20 μg/ml in PBS) was added to each tissue culture plate and incubated at 4°C overnight. The following day, the fibronectin solution was removed and plates were incubated with DMEM containing 0.5 mg/ml BSA in 5% CO_2 atmosphere at 37°C for 30 min to block non-specific binding. Medium was aspirated, and cells previously in suspension were then plated and returned to the incubator for the times indicated.

SDS–PAGE AND WESTERN BLOTTING

Cell cultures were placed on ice and washed with 5 ml of ice cold PBS (with 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$—referred to as PBS*). Cells were lysed in 400 μl of cold RIPA buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% ultra pure sodium dodecyl sulfate, 50 mM Tris, 1% Nonidet P-40 and 10 mM EDTA, pH 7.4) with inhibitors 1 mM Na_3VO_4, 10 mM NaF, 2 μg/ml aprotinin and 2 μg/ml leupeptin and 1 mM PMSF, and were removed with a cell scraper. For isolation of the cytoskeletal fraction, a two step fractionation was performed. Cells were extracted for 1 min in 500 μl of ice cold Csk buffer (50 mM MES, 3 mM EGTA, 5 mM $MgCl_2$, 0.5% Triton X-100, pH6.4). Csk buffer solubilizes cytosolic, microsomal and some plasma membrane components of the cells. The remaining material on the plate was then rinsed quickly in 500 μl Csk buffer, and extracted with 500 μl RIPA buffer. This material constitutes the insoluble cytoskeletal fraction. All buffers were supplemented with protease and phosphatase inhibitors as indicated above.

For non-adherent conditions, cell suspensions were transferred to 15 ml conical tubes and centrifuged at 1,100 rpm in an IEC HN-S centrifuge for 5 min. Supernatants were then removed and pelleted cells were washed with 1 ml of ice-cold PBS*, and lysed with 500 μl of RIPA buffer. Protein determination was performed using Bio-Rad Dc Protein Assay reagents (Bio-Rad), and equal protein sample amounts were loaded.

For immunoprecipitations, antibodies and protein A sepharose beads were added to lysates, and incubated together on a rotator at 4°C overnight. Immunoprecipitates were then washed three times, each with 1 ml of RIPA buffer, and centrifuged at 14,000 rpm for 10 min in an IEC/Micromax centrifuge at 4°C between each wash. For co-immunoprecipitation experiments, cells were lysed with NP-40 buffer (1% Nonidet P-40, 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, pH 7.4). Immunoprecipitation with rat anti-α5 and anti-β1 antibodies was carried out as above, and anti-rat IgG-coated agarose beads were used for pull down. Whole cell lysates and immunoprecipitates were subjected to 8% reducing SDS–PAGE, followed by semi-dry transfer of proteins to polyvinylidene fluoride (PVDF) membranes.

For Western blotting, membranes were blocked with 5% BSA or 5% milk powder in Tris-Buffered saline Tween-20 (TBST) for 1 h, followed by primary antibody incubations overnight. Blots were then washed three times in TBST, each for 10 min, followed by secondary HRP antibody incubations at 1:2,500 in TBST for 1 h. Membranes were washed three times in TBST, each for 10 min. Blots were then incubated for 1 min in Western Lightning chemiluminescence reagents (Amersham Biosciences) and exposed to autoradiograph film. In some experiments (Fig. 2C), band intensities were assessed using Corel Photopaint software, and the ratios of pYMet/PanMet and pYSrc/PanSrc were calculated and expressed as a percentage of the untreated control corresponding to each cell line.

IMMUNOFLUORESCENCE

Indirect immunofluorescence staining was carried out as described previously [Elliott et al., 2004]. Briefly, cells were plated overnight on cover slips, fixed in 3% paraformaldehyde/PBS*, permeablized with 0.2% Triton X-100, and blocked for 30 min with 3% BSA. Cells were incubated with the indicated antibodies, followed by the appropriate secondary antibody. Preparations were viewed using a Leica TCS SP2 confocal microscope in the Queen's Cancer Research Institute and Protein Discovery and Function Facility. Co-localization was assessed using ImagePro software (Symbol Technologies, Mississauga, ON).

CELL SPREADING ASSAY

Cells were incubated overnight, and replated (10^4 cells in 1 ml/well) on fibronectin-coated 24 well tissue culture plates in serum free medium as described above. Cells were incubated for varying times up to 90 min, and fixed in 3% paraformaldehyde/PBS, and stained with Toluidine Blue (0.5% in PBS). Four images per well were photographed at 20× under a Leica inverted microscope with phase contrast optics, and images were assessed for rounded (bright phase contrast halo with no extensions) versus spread (flat or slightly rounded with extensions, no halo). A total of three wells per group are counted and plotted as the average % cell spreading ± standard error.

RESULTS

SRC INDUCES MET ACTIVATION IN EPITHELIAL CELLS IN AN HGF-INDEPENDENT MANNER

Src has been reported to phosphorylate specific tyrosine residues in the activation loop of some RTKs thereby stabilizing activation of the receptor independently of ligand [Danilkovitch-Miagkova et al., 2000; Moro et al., 2002]. In order to assess the ability of Src to activate Met independently of HGF, we expressed an activated Src mutant in the non-neoplastic breast epithelial cell line HC11, which

expresses Met but not HGF [Wojcik et al., 2006]. Cells were allowed to adhere for 60 min on fibronectin substratum, and assessed for the phosphorylation status of signaling proteins by Western blotting analysis, or for cell spreading phenotype (Fig. 1). HC11 cells expressing activated Src showed a marked increase in Met activation, as demonstrated by western blotting with antibody against phospho-tyrosine residues pY1230, 1234, and 1235 in the activation loop of Met (referred to as pYMet), compared to the parental cells (Fig. 1A). Strong phosphorylation of Y397 of FAK in HC11 cells both with and without activated Src was also observed, consistent with engagement of the cell–matrix adhesion signaling cascade. Phosphorylation of FAK at Y407, Y861, and Y925, known phosphorylation sites of Src, was markedly elevated in HC11 cells expressing activated Src, confirming Src activity. As expected, activated Src caused a marked increase in spreading of HC11 epithelial cells on fibronectin substratum (Fig. 1B).

To determine if endogenous Src is required for stabilization of pYMet in adherent cells, we examined the level of endogenous Met phosphorylation in SYF−/− embryonic fibroblasts (lacking Src, Yes, and Fyn) compared to corresponding wild-type (SYF+/+) cells following adhesion to fibronectin substratum. Significant tyrosine phosphorylation of Met was present in wild-type (SYF+/+) fibroblasts, was absent in SYF−/− cells, but was rescued in SYF−/− + Src cells in which c-Src was added back, as determined by western blotting (Fig. 2A,B).

Together these findings indicate that activated Src can induce tyrosine phosphorylation of Met in epithelial cells, and that Src is required for stabilization of pYMet in adherent cells.

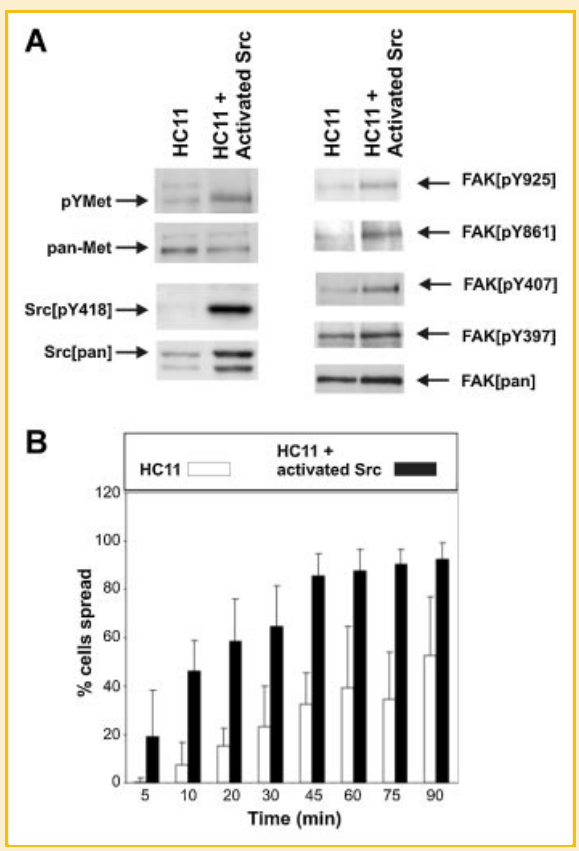

Fig. 1. Activated Src promotes Met phosphorylation in breast epithelial cells: (Panel A) HC11 breast epithelial cells alone, or expressing activated Src, were incubated overnight in 10 cm diameter tissue culture plates at 7.5×10^5 cells/plate, and lysed in 1× Laemmli buffer. Equal protein amounts were subjected to 10% SDS–PAGE under reducing conditions, and Western blotting with the indicated antibodies was performed. Panel B: HC11 cells alone, or expressing activated Src, were plated on fibronectin substratum for various times and assessed for cell spreading as described in Materials and Methods Section.

HGF-INDUCED MET ACTIVATION OCCURS INDEPENDENTLY OF SRC FAMILY KINASES IN EPITHELIAL CELLS

To determine if Met is capable of responding to exogenous HGF in the absence of Src activity, HC11 cells alone, or expressing activated Src were treated overnight with the Bcr-Abl/Src family kinase inhbitior Dasatinib [Shor et al., 2007]. Cells were then stimulated with recombinant HGF (120 ng/ml) for 20 min and assessed for pYMet levels by Western blotting (Fig. 2C). As expected, treatment with Dasatinib at concentrations from 0.1 to 1.0 μM resulted in a dose-dependent inhibition of pY418Src, concomitant with a decrease in pYMet to baseline levels. Remarkably, parental HC11 cells treated with 0.3 μM Dasatinib showed an HGF-induced ~20-fold increase in the ratio of pYMet/PanMet, while inhibition of pY418Src was maintained. Similarly, HC11 cells expressing activated Src showed a ~70-fold increase in the pYMet/PanMet ratio compared to drug-treated (1.0 μM) cells alone. A concomitant ~3-fold reduction in pan Met also occurred in HGF-treated cells, possibly representing receptor turnover [Niendorf et al., 2007]. Interestingly, HGF did not rescue pYMet in SYF−/− fibroblasts (Fig. 2B). Together, these results show that Dasatinib has no detectable effect on HGF-induced Met phosphorylation, indicating that HGF-dependent activation of Met in breast epithelial cells can occur in the absence of Src family kinase activity. Furthermore, HGF-dependent Met activation occurs preferentially in epithelial cells, with minimal effect of HGF on Met activity in fibroblasts.

SRC PROMOTES MET ACTIVATION AND CELL SPREADING IN MAMMARY CARCINOMA CELLS

We have previously shown that endogenous active Src acts through Stat3 to stimulate HGF transcription in a murine breast carcinoima cell line, SP1, thereby promoting an autocrine HGF/Met loop [Hung and Elliott, 2001; Wojcik et al., 2006]. We therefore examined the effect of Src on Met activation levels in SP1 cells. As expected, expression of an activated Src mutant causes an increase in the steady state level of pYMet compared to untransfected cells (Fig. 3A). Conversely, inhibition of Src activity with a dominant negative, kinase dead Src (Src-RF) mutant reduces pYMet in SP1 cells. In addition, cells expressing kinase dead Src showed a marked reduction in cell spreading on fibronectin substratum, compared to the parent cells, or cells expressing activated Src (Fig. 3B). Similarly, treatment with a Src family kinase inhibitor PD180970 [Garcia et al., 2001] results in a marked dose-dependent reduction (up to 65%) in pYMet within 12 h (Fig. 3C). A similar reduction of pYMet was observed with the Dasatinib inhibitor (Fig. 2C, and data not shown). Interestingly, pYMet is primarily localized to the plasma membrane in SP1 cells, particularly in the leading filopodia extensions (Fig. 3D), characteristic of a cell scattering phenotype. Moreover,

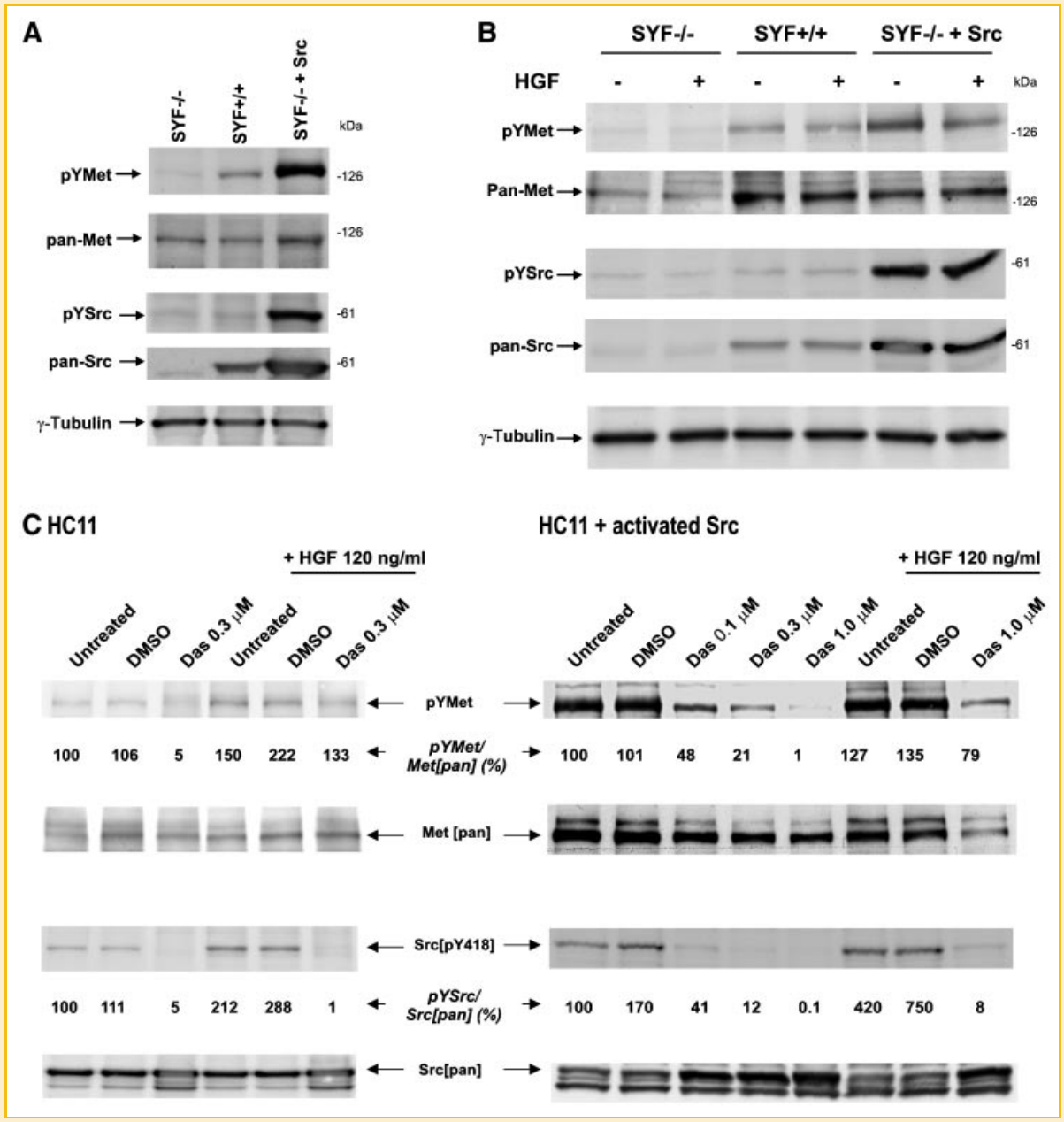

Fig. 2. Src is required for adhesion-dependent, but not HGF-induced, Met activation: (Panel A) SYF−/−, SYF+/+ and SYF−/− + Src embryonic fibroblasts were subcultured overnight and plated on fibronectin substratum as described in Figure 1. Cells were lysed in 1× Laemmli buffer, and equal protein amounts were subjected to 10% SDS–PAGE under reducing conditions. Western blotting was performed with the indicated antibodies. Panel B: SYF−/−, SYF+/+, and SYF−/− + c-Src embryonic fibroblasts were subcultured overnight and stimulated with DMSO vehicle alone, or rHGF (120 ng/ml) for 20 min, lysed and subjected to SDS–PAGE and Western blotting as in Panel A. Panel C: HC11 cells (left) were cultured for 12 h with DMSO/DMEM (7% FBS) alone or with the Dasatinib inhibitor at the concentrations indicated. After a further incubation (6 h) in serum-free medium with the same treatments, rHGF (120 ng/ml) was added to the indicated cultures, and cells were incubated for an additional 20 min. In a parallel experiment, HC11 cells expressing activated Src (right) were treated with the same protocol, but without serum starvation. Western blotting was carried out as in Panel A with the indicated antibodies. Densitometry of band intensities was carried out as described in Materials and Methods Section; the ratios of pYMet/PanMet and pYSrc/PanSrc were calculated, and expressed as percentages normalized to the corresponding untreated group in each cell type.

treatment with the above Src family kinase inhibitors causes a marked reduction in Met phosphorylation and filopodia retraction (Fig. 3D, and data not shown). Thus, SP1 carcinoma cells that secrete HGF still require Src for full Met activation, consistent with a role of Src in regulating autocrine HGF production as well as ligand-independent activation of Met.

CELL–MATRIX ADHESION IS REQUIRED FOR SRC-DEPENDENT MET ACTIVATION IN MAMMARY EPITHELIAL AND CARCINOMA CELLS

Cell–matrix adhesion has been shown to be required for interaction between Src and its substrate FAK, and phosphorylation of FAK by Src [Lin et al., 2004]. We therefore examined the effect of cell–matrix adhesion on pYMet in mammary carcinoma and epithelial

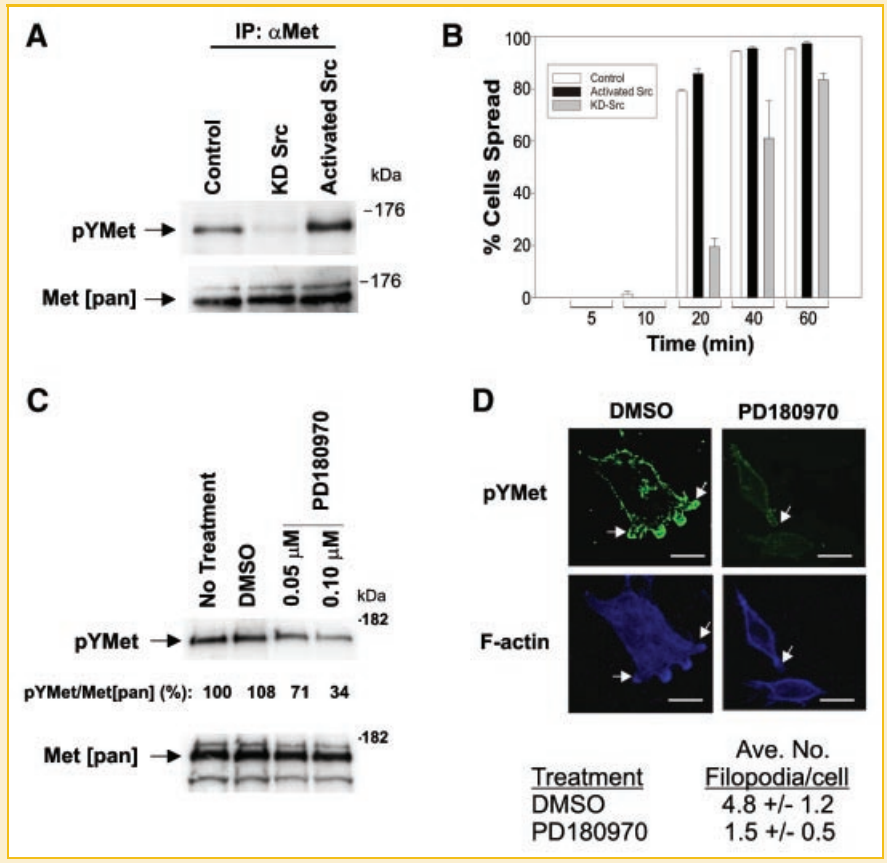

Fig. 3. Inhibition of Src activity blocks constitutive Met phosphorylation in breast carcinoma cells. Panel A: SP1 carcinoma cells alone, or expressing a kinase dead Src mutant, or an activated Src mutant (Y527F), were serum-starved overnight. Cells were washed with PBS*, lysed using RIPA buffer, and immunoprecipitated with anti-Met antibody. Equal protein amounts from whole cell lysates and Met immunoprecipitates of each cell type were resolved on parallel 10% SDS–PAGE gels, followed by Western blotting with anti-Met[pY1230,1234,1235] (subsequently referred to as pYMet) and anti-Met[pan]. Data shown are representative of three experiments. Panel B: SP1 cells expressing wild-type and mutant Src constructs from Panel A were plated on fibronectin substratum for various times and assessed for cell spreading as described in Materials and Methods Section. Panel C: SP1 cells were plated at 4.0×10^5 cells per 5 cm diameter tissue culture plate in DMEM with 10% FBS. Cells were subjected to the following conditions in complete medium for 12 h: No treatment, DMSO alone, 0.05 μM PD180970, and 0.1 μM PD180970. Cells were then lysed in RIPA buffer, and equal protein amounts were resolved by 8% SDS–PAGE. Western blotting with anti-pYMet and anti-Met[pan] antibodies was performed. Optical densitometric ratios of pYMet to total Met in each lane are shown as a percentage of the no treatment control. Panel D: SP1 cells were grown on coverslips overnight, then treated with DMSO alone or with 0.1 μM PD180970 for 12 h. Immunofluorescence staining was performed with anti-pYMet detected by Alexa 488-labeled anti-rabbit IgG (green), and Alexa 350-labeled phalloidin (blue). The average number of pseudopodia per cell was determined by visual assessment of confocal images from random fields in each group (N = 20 cells per group). Results in Panels C and D are representative of two experiments.

cells. We observed that the level of tyrosine phosphorylation of Met was markedly reduced in SP1 carcinoma cells expressing activated Src cultured in suspension for 2 h, but was fully recovered following re-attachment to, and spreading on, a fibronectin substratum for 1 h (Fig. 4A). In addition, Src-induced Met phosphorylation in non-neoplastic HC11 epithelial cells (which do not express HGF) was reduced to baseline within 15 min of cell detachment, and remained so for up to 90 min in cells in suspension (Fig. 4B). However, Met phosphorylation in these cells was restored within 10 min of re-adhesion to a fibronectin substratum (Fig. 4C). For both carcinoma cells and Src-transformed epithelial cells, a high level of Src activity (indicated by phosphorylation at Src[Y418]) was maintained for up to 90 min following cell detachment (Fig. 4A,B) as previously reported [Lin et al., 2004]. These findings demonstrate that Src-induced activation of Met is tightly linked to cell–matrix adhesion through a rapid signaling mechanism both in carcinoma cells that express HGF and in epithelial cells that do not.

PHOSPHORYLATED MET CO-LOCALIZES WITH $\alpha 5\beta 1$ INTEGRINS AND REQUIRES F-ACTIN ASSEMBLY IN CELLS EXPRESSING ACTIVATED SRC

Since previous studies have indicated that some RTKs (e.g. EGFR) associate with integrins and Src in the focal adhesion complex [Moro et al., 1998; Bromann et al., 2004], we examined whether Met is complexed with integrins in SP1 carcinoma cells expressing an activated Src mutant. Cells were lysed in 1% NP-40 buffer which was shown to solubilize the majority of Met protein (not shown), and immunoprecipitated with antibodies against α5 and β1 integrins (Fig. 5A). The results showed that significant amounts of total Met and pYMet proteins are present in both immunoprecipitates as determined by Western blotting. Controls showed reciprocal pull

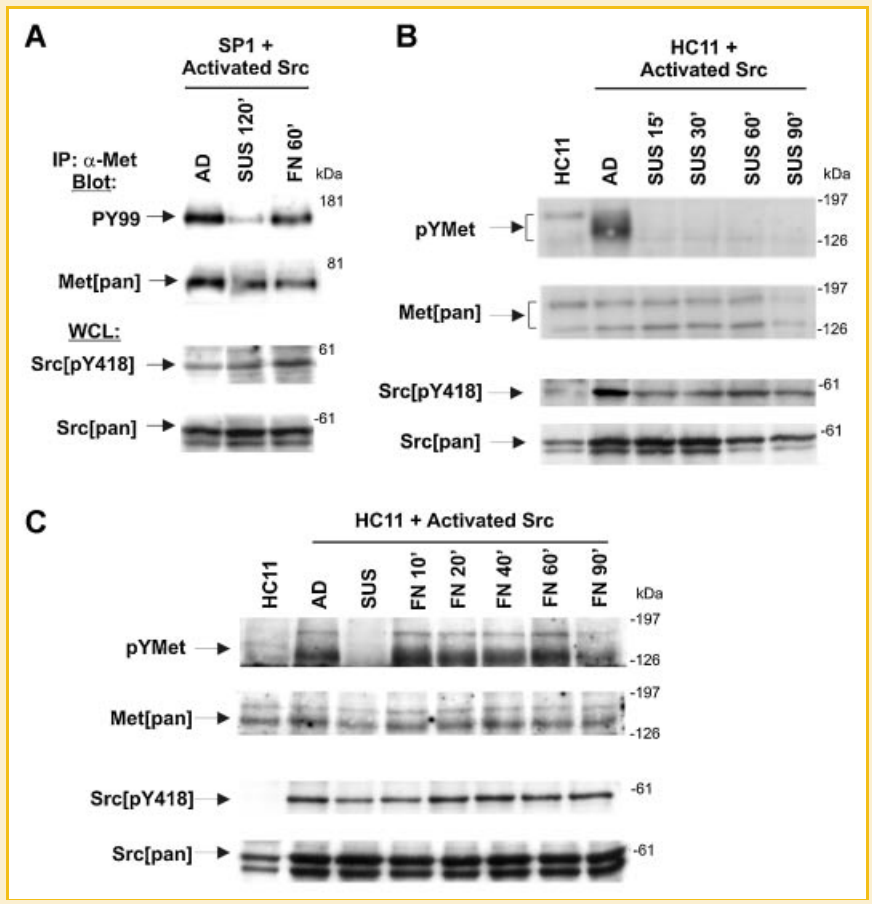

Fig. 4. Src-dependent Met phosphorylation requires cell matrix adhesion. Panel A: SP1 cells expressing activated Src were serum starved overnight, and subcultured in DMEM medium with 0.5 mg/ml of BSA under the following conditions: adherent (AD) on plastic, in suspension (SUS) for 2 h, and re-plated on fibronectin (FN) substratum (20 μg/ml) for 1 h. Cells from each group were lysed with cold RIPA buffer. Equivalent protein samples were used for Met immunoprecipitation. Samples were resolved on parallel 8% SDS–PAGE gels and transferred onto membranes which were probed for phosphorylated tyrosine (PY99) and Met[pan]. A parallel western blot of corresponding whole cell lysates (WCL) was probed for Src[pY418] and Src[pan]. Results are representative of three experiments. Panel B: HC11 mammary epithelial cells alone, or expressing activated Src were incubated overnight in complete DMEM medium. Next day, cells were harvested in trypsin/EDTA PBS, resuspended in serum-free medium with 0.5 mg/ml of BSA, and incubated in suspension for the times indicated. At each time point, cells were lysed in 1× Laemmli buffer, and equal protein amounts were subjected to 10% SDS–PAGE under reducing conditions. Western blotting was performed with the indicated antibodies. Panel C: HC11 cells expressing activated Src were incubated in suspension for 20 min, and then replated on fibronectin-coated plates (20 μg/ml) for the times indicated. At each time point, cells were lysed and equal protein amounts were subjected to 10% SDS–PAGE under reducing conditions and western blotting as indicated in Panel B. The results are representative of two experiments.

down of the α5 chain with β1 integrin, while very little material was pulled down by beads alone. Double immunofluorescence staining showed strong co-localization of pYMet with both β1 integrins and vinculin, consistent with association of the pYMet/integrin complex with focal adhesions (Fig. 5B).

Actin polymerization is an important requirement for activation of Src and its localization to focal adhesions [Sandilands et al., 2004]. We therefore examined the effect of latrunculin A, which sequesters G-actin monomers [Morton et al., 2000], on Met phosphorylation. Treatment of activated Src-expressing HC11 cells with latrunculin A caused dephosphorylation of Met within 10 min; rephosphorylation was detected within 30 min and was completely restored within 2 h following washing with fresh medium (Fig. 6A). The level of phosphorylation of Src[Y418], though partially reduced, remained elevated in latrunculin A-treated cells compared to parental HC11 cells. Immunofluoresence analysis showed a similar decrease in phosphorylated Met concomitant with a loss of F-actin filaments in latrunculin A treated, compared to untreated cells (Fig. 6B). Likewise, rephosphorylation of Met along with reorganization of F-actin filaments occurred within 2 h following washing. Interestingly, pYMet was co-localized with F-actin, particularly at adhesion sites. Thus, actin polymerization is required for Src-induced phosphorylation of Met, and its localization with focal adhesions.

SRC-PHOSPHORYLATED MET IS ASSOCIATED WITH THE CORTICAL CYTOSKELETON

Since pYMet is localized to focal adhesions and requires F-actin assembly (Figs. 5 and 6), we examined whether Src-dependent phosphorylation of pYMet is associated with the cortical cytoskeleton. To test this possibility, we separated soluble (cytosolic) and insoluble (cytoskeleton-associated) cellular fractions using 0.5% Triton X-100 (Csk buffer) from HC11 cells expressing activated Src compared to parental cells (which exhibit low Src activity)

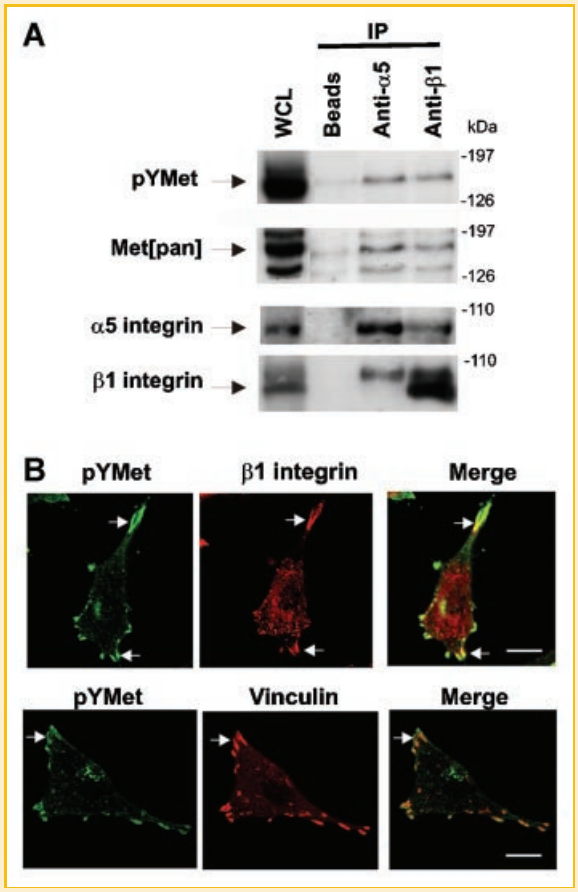

Fig. 5. Phosphorylated Met is associated with α5β1 integrins in the focal adhesion complex: (Panel A) SP1 cells expressing activated Src were sub-cultured (2.0×10^6) in 10 cm diameter dishes in DMEM with 10% FBS, and serum starved overnight as in Figure 3. Cells were lysed using 1% NP-40 buffer, and equal amounts of protein were immunoprecipitated with α5 or β1 integrin rat monoclonal antibodies, and anti-rat IgG-coated agarose beads. Controls consisted of whole cell lysate alone or with beads only. Immunoprecipitates were split and resolved on parallel 8% reducing SDS–PAGE gels, and western blotting was performed using antibodies against pYMet, Met[pan] α5 and β1 integrins. Panel B: Cells from Panel A were plated overnight on glass coverslips, fixed, and double immunofluorescence staining for pYMet and β1 integrin (upper) or vinculin (lower) was carried out. Results are representative of two experiments.

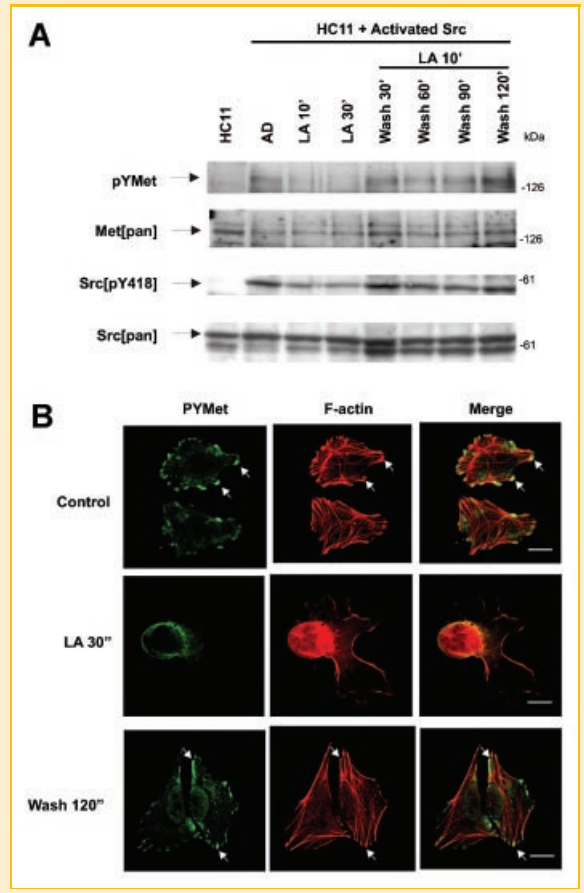

Fig. 6. Treatment with latrunculin A blocks Met phosphorylation in HC11 cells expressing activated Src: Wild-type HC11 cells alone, or expressing activated Src were incubated overnight in 10 cm diameter tissue culture plates at 7.5×10^5 cells/plate. Next day, cells were incubated with latrunculin A (LA, 1 μM) in complete medium for 10 or 30 min. After 30 min, cells in some groups were washed, and allowed to incubate in fresh medium for an additional time period as indicated. Panel A: At each time point, cells were lysed in 1× Laemmli buffer, and equal protein amounts were subjected to 10% SDS–PAGE under reducing conditions. Western blotting was performed with antibodies against pYMet, Met[pan], Src[pY418] and Src[pan]. Panel B: HC11 cells expressing activated Src were cultured overnight on coverslips, and cells were treated with DMSO alone, or with latrunculin A (1 μM) for 30 min, and then washed with fresh medium for 2 h. Cells in the indicated groups were fixed and immuno-fluorescence staining with anti-pYMet (Alexa-488, green), or F-actin (TRITC-phalloidin, red) was carried out. The results are representative of at least two experiments.

(Fig. 7A). Fractionated proteins were solubilized in loading buffer and analyzed by Western blotting with the antibodies indicated. HC11 cells expressing activated Src showed a strong increase in pYMet with very little change in total Met associated with the insoluble (cytoskeleton-associated) fraction compared to parental HC11 cells, consistent with a role of Src in the tyrosine phosphorylation of Met at the cytoskeleton. These cells also showed significant Src[pY418] and vinculin associated with the insoluble fraction. Enrichment of actin in the insoluble fraction in both cell lines confirmed the efficiency of the extraction process. Immuno-fluorescence analysis showed a strong co-localization of pYMet with activated Src and vinculin at focal adhesions in HC11 cells expressing activated Src (Fig. 7B). In contrast, a diffuse low level of pYMet and vinculin expression was detected in HC11 cells expressing empty vector (Fig. 7C). Co-localization of pYMet with Src and F-actin at focal adhesions was also detected in cells following extraction with Csk buffer, confirming the presence of the pYMet/Src complex in the cytoskeletal fraction (Fig. 7D). These findings indicate that Src-induced pYMet is colocalized with focal adhesions and the actin cytoskeleton in epithelial cells.

FAK IS REQUIRED FOR SRC-INDUCED MET PHOSPHORYLATION IN BREAST CARCINOMA CELLS

Src interacts with β1 integrins indirectly through its substrate FAK [Avizienyte and Frame, 2005; McLean et al., 2005], and FAK is required for Src-dependent cell spreading and migration

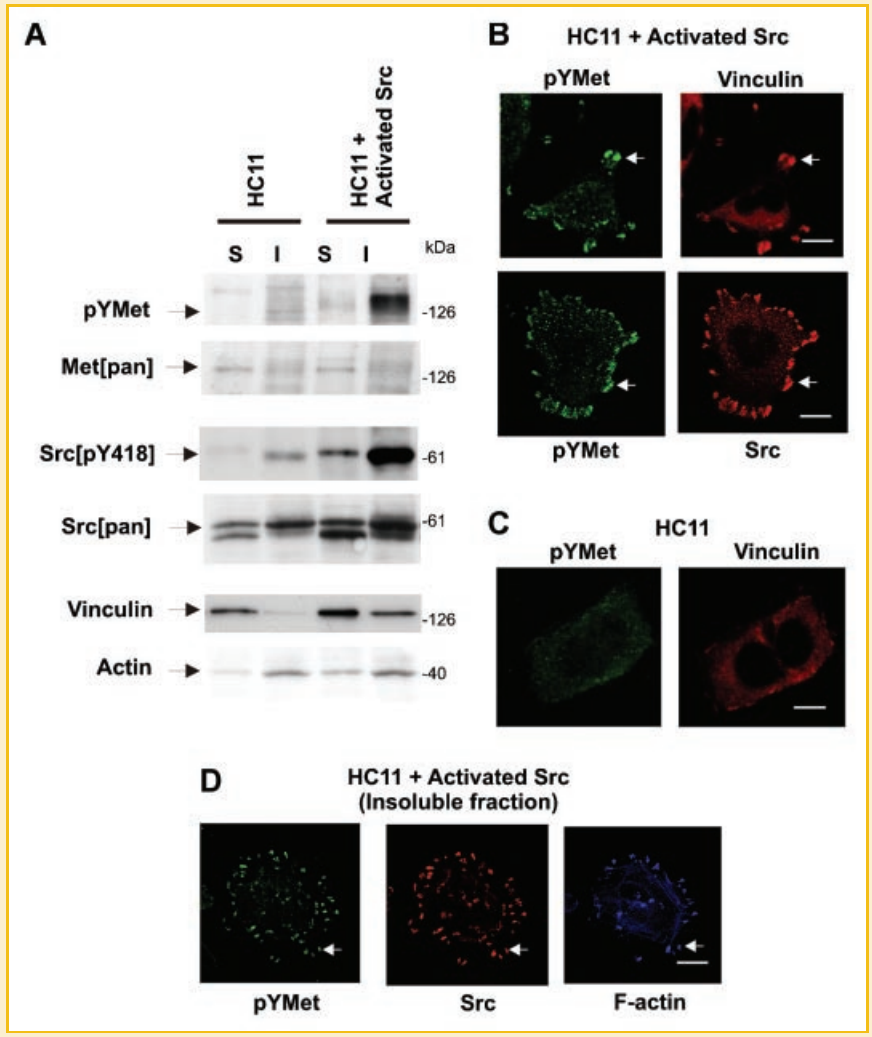

Fig. 7. pYMet is associated with the cytoskeletal fraction in HC11 cells expressing activated Src. Panel A: HC11 mammary epithelial cells alone, or expressing activated Src were incubated overnight in 10 cm diameter tissue culture plates at 7.5×10^5 cells/plate. Next day, cells were extracted with 500 μl Csk buffer (soluble, S) for 1 min on ice. The remaining insoluble (I) material was rinsed quickly with 500 μl Csk buffer, and then extracted with 500 μl RIPA buffer. Samples were made up to equal volumes with 6× Laemmli buffer, and protein concentration was determined. Equal protein amounts were subjected to reducing 10% SDS–PAGE. Western blotting was performed with the antibodies indicated. Results are representative of three experiments. Panel B: HC11 cells expressing activated Src were plated on coverslips overnight in complete medium, and double immunofluorescence staining was carried out as described in Materials and Methods Section, with antibodies against pYMet and activated Src (upper images), or pYMet and vinculin (lower images). Panel C: HC11 cells were plated overnight on coverslips in complete DMEM medium, and double staining with antibodies against pYMet and vinculin was performed. Panel D: HC11 cells expressing activated Src were plated on coverslips as above, treated with Csk buffer, followed by triple staining with antibodies against pYMet, vinculin, and phalloidin. Results are representative of two experiments.

[Richardson et al., 1997]. We therefore examined the role of FAK in cell matrix adhesion-induced Met phosphorylation using two approaches. First, we examined the level of endogenous Met phosphorylation in FAK−/− embryonic fibroblasts compared to the corresponding wild-type cells following adhesion to fibronectin substratum. FAK−/− fibroblasts showed a marked reduction in pYMet compared to corresponding wild-type cells (Fig. 8A), thus indicating a requirement for FAK-dependent signaling in stabilizing pYMet in adherent cells.

Second, we used a dominant negative mutant of FAK (FAK407-925F), with phenylalanine substitutions at five tyrosines (Y407F, Y576F, Y577F, Y861F, and Y925F) known to be phosphorylated by Src, and which can block cell scattering while restoring cell–cell contacts in carcinoma cells expressing activated Src [Avizienyte et al., 2002]. Over-expression of the Myc-tagged FAK407-925F mutant in HC11 epithelial cells expressing activated Src inhibited Met phosphorylation and its localization in the insoluble fraction, compared to cells expressing wild-type FAK or vector only (Fig. 8B). Cells expressing the FAK407-925F construct showed reduced phosphorylation of FAK at tyrosines corresponding to the mutated sites compared to wild-type FAK, indicating the dominant negative effect of this mutant (Fig. 8C). Strong co-localization of wild-type FAK with pYMet at focal adhesions was observed (Fig. 8D). In contrast, cells expressing the FAK407-925F mutant showed rounded cell morphology with increased cell–cell contacts and weak diffuse pYMet staining. These findings indicate that phosphorylation of

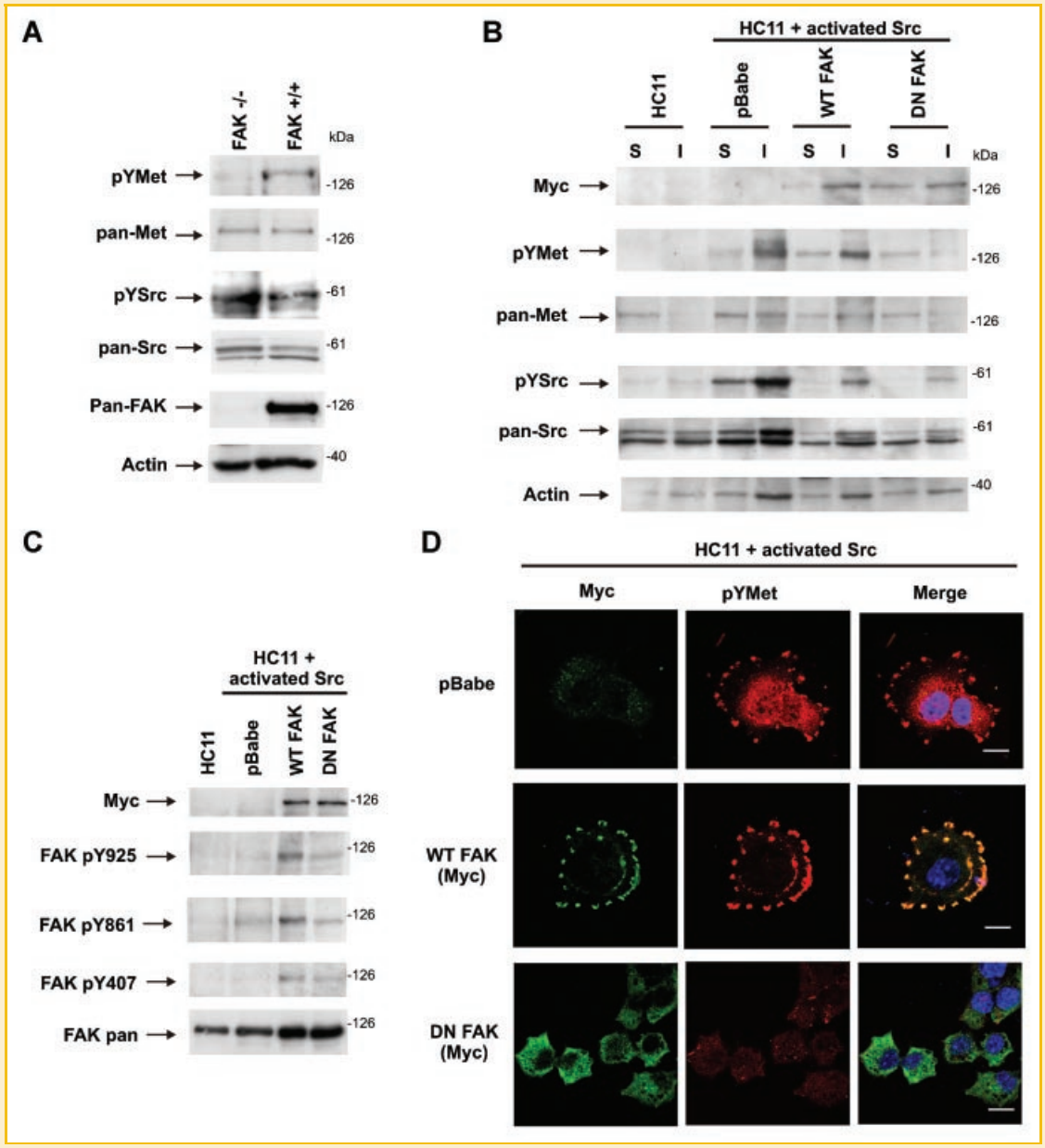

Fig. 8. Src-induced phosphorylation of Met requires interaction of Src with FAK: (Panel A) FAK+/+ and FAK−/− embryonic fibroblasts were subcultured as described in Panel A. Cells were then lysed and subjected to 10% SDS–PAGE under reducing conditions and Western blotting was performed with the indicated antibodies. Panel B: Wild-type HC11 cells expressing activated Src were co-transected with Myc-tagged wild-type FAK or a dominant negative FAK mutant (FAK407-925F) incubated overnight in complete medium and plated on fibronectin substratum for 1 h as in Figure 1. Triton X 100 soluble and insoluble fractions were harvested and equilibrated with 1× Laemmli buffer as in Figure 7A. Equal protein amounts from each fraction were subjected to 10% SDS–PAGE under reducing conditions. Western blotting was performed with the indicated antibodies. Panel C: Whole cell lysates from Panel B were subjected to 10% SDS–PAGE under reducing conditions. Western blotting was performed with the indicated phospho-FAK, pan FAK, and Myc-tag specific antibodies. Panel D: Cells from panel AQ were plated overnight on coverslips in complete DMEM medium, and immunofluorescence staining was performed with the indicated antibodies. Results are representative of two experiments.

FAK by Src is an important step in cell adhesion-dependent Met activation and cell spreading.

DISCUSSION

Previous reports, indicating that cell–matrix adhesion can promote ligand-independent activation of Met [Wang et al., 1996, 2001; Rusciano et al., 1995, 1998, 1999] and is required for Src-dependent tyrosine phosphorylation of FAK [Lin et al., 2004], prompted us to examine the possible role of Src in Met activation. In this report, we show that a Src/FAK interaction network and intact actin cytoskeleton are required for cell–matrix adhesion-dependent activation of Met.

To assess HGF-independent effects of Src on Met activation we used a model of Src-induced transformation in a breast epithelial

cell line, HC11, which expresses Met but not HGF [Wojcik et al., 2006]. We demonstrated that activated Src promotes a strong phosphorylation of tyrosines in the autoactivation loop of Met (pYMet) in HC11 cells, and that this effect is highly dependent on cell–matrix adhesion. As predicted, treatment with Dasatinib, a Bcr-Abl/Src family kinase inhibitor, caused a reduction in pYMet and filopodia extensions. Cell adhesion-induced pYMet expression was also abrogated in SYF−/− fibroblasts compared to wild-type or SYF−/− + c-Src cells (with add-back of c-Src). Together, these findings suggest a positive regulatory role of Src on Met activation in breast epithelial and fibroblast cells, similar to that observed for other RTKs [Peterson et al., 1996; Danilkovitch-Miagkova et al., 2000].

To directly assess the role of Src in HGF-dependent activation of Met, we examined the effect of the Dasatinib inhibitor on HGF-stimulated Met in HC11 cells alone, or expressing activated-Src, both of which are HGF-negative. Notably, rescue of pYMet levels by HGF was seen in both cell lines, indicating that HGF-induced Met activation occurs largely independently of Src family kinases, as described for PDGF-induced signal transduction [Klinghoffer et al., 1999]. Interestingly, addition of exogenous HGF under the same conditions did not activate Met in SYF−/− or wild-type embryonic fibroblasts, indicating a primarily HGF-independent (Src-dependent) mechanism of Met activation in these cells. These results suggest that HGF-dependent and HGF-independent pathways of Met activation occur via distinct mechanisms, depending on cell type and tissue microenvironment.

We previously demonstrated that HGF is frequently co-expressed with Met in human invasive breast cancers, consistent with a role of autocrine HGF/Met signaling [Tuck et al., 1996]. Furthermore, we showed that Src acts co-operatively with Stat3 to activate HGF transcription in both mouse and human breast carcinoma cells in monolayer cultures [Wojcik et al., 2006; Sam et al., 2007]. In the present study, we therefore examined the effect of Src on Met activation in a mammary carcinoma cell line, SP1, which expresses both HGF and pYMet [Rahimi et al., 1996]. We showed that the steady state level of pYMet is reduced in SP1 cells following pharmacological or genetic inhibition of Src, consistent with a role of Src in regulating an autocrine HGF/Met loop in these cells. Interestingly, cell–matrix adhesion still positively regulates pYMet in SP1 cells, supporting a secondary role of integrin-based adhesion in stabilizing Met activity, as described by Wang et al. [2001].

Our demonstration of rapid dephosphorylation of Met following detachment of breast epithelial or carcinoma cells expressing activated Src indicates that the integrin requirement in the activation of Met is independent of autocrine expression of HGF or other growth substances. A similar dependency on cell–matrix adhesion of activated Src to bind to, and phosphorylate, its substrate FAK[Y577] was previously observed [Lin et al., 2004]. In addition, integrin engagement is required for deregulation of cadherin-based cell–cell contacts by activated Src [Avizienyte et al., 2002]. Interestingly, we have previously shown that HGF restores tyrosine phosphorylation of Met and stimulates a PI3K/Akt-dependent survival pathway in serum-starved detached SP1 carcinoma cells [Qiao et al., 2000]. This effect was independent of Src kinase activity, similar to our observed rescue by exogenous HGF of Met activation in Dasatinib treated cells in the present study. Thus, depending on the ECM microenvironment, both paracrine and autocrine effects of HGF have been observed in SP1 carcinoma cells. Together these findings link Src and integrin signaling in an HGF-independent pathway of Met activation, and identify an HGF-dependent Src/integrin-independent pathway of Met activation in epithelial and carcinoma cells.

A novel aspect of our study is that Src-induced pYMet requires interaction of Src with its substrate FAK. We showed that pYMet is markedly reduced in FAK−/− compared to corresponding wild-type embryonic fibroblasts which express endogenous levels of pYMet and Src[pY418]. To identify putative Src-FAK protein-protein interactions in the regulation of pYMet we used a dominant negative FAK mutant (FAK407-925F), lacking all of the known Src-dependent phospho-acceptor sites (Y407, Y407, Y577, Y861, Y925 are mutated to phenylalanine) [Avizienyte et al., 2002]. The FAK407-925F mutant markedly reduces Met activation concomitant with cell rounding and retraction of pseudopodia extensions. These results mirror a previous report that FAK is required in integrin-ErbB2/3 cross talk in the regulation of invasion of carcinoma cells [Benlimame et al., 2005], and that interaction of FAK with Met is required for HGF-induced cell invasion [Chen and Chen, 2006]. Our results thus identify a key role of the Src/FAK interaction network in cell–matrix adhesion-dependent activation of Met and cell scattering.

Since actin polymerization and focal adhesion turnover are important cellular functions regulated by Src and FAK [Frame, 2004; Sandilands et al., 2004], we examined the role of actin cytoskeleton in stability of pYMet by Src and the association of pYMet with focal adhesions. Similar to the cell adhesion effect, a rapid, reversible dephosphorylation of Met (within 10 min) following disruption of actin polymerization with Latrunculin A in epithelial cells expressing activated Src was observed. Furthermore, using co-localization and cell fractionation approaches we showed that phosphorylated Met is associated with both focal adhesions and actin filaments near the plasma membrane, supporting a role of phosphorylated Met in cell migration and invadapodia formation as has been shown for activated Src [Hauck et al., 2002].

The mechanism by which the Src/FAK axis regulates cell–matrix-dependent Met phosphorylation is not known. One possibility is that co-aggregation of Met and Src/FAK with integrins at the plasma membrane facilitates direct phosphorylation of Met by Src, as indicated for PDGF-β receptor [Sundberg and Rubin, 1996; Thomas and Brugge, 1997]. Another possibility is that intermediary kinases or adaptor proteins (e.g., p130cas and paxillin) are recruited to the focal adhesion complex and act downstream of Src and FAK to indirectly mediate phosphorylation of Met, as demonstrated for the EGF receptor [Moro et al., 2002] and Flt-1 [Hanks et al., 2003].

The inhibitory effect of Dasatinib on pYMet is particularly interesting as this inhibitor has been shown to block Bcr-Abl/Src family kinase activity in soft tissue tumors [Shor et al., 2007]. Furthermore, cancer cell lines corresponding to the basal-like breast cancer subset, which is extremely resistant to conventional therapies, have been shown to be highly sensitive to Dasatinib

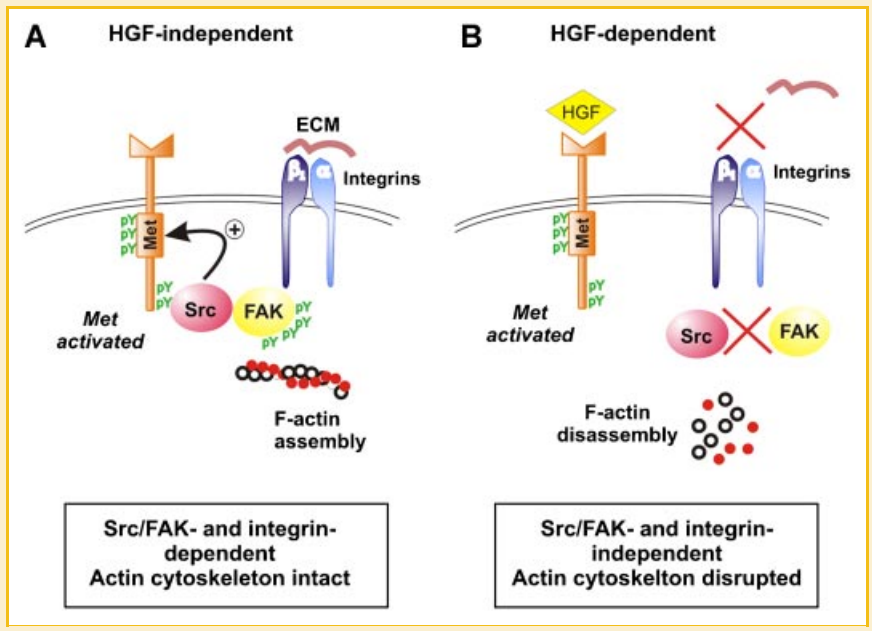

Fig. 9. Proposed models of Met activation: (Panel A) HGF-independent: Activation of Src and phosphorylation of FAK by Src causes phosphorylation of Met at tyrosine residues in the activation loop. This process is highly dependent on integrin-based cell adhesion and requires an intact actin cytoskeleton. Panel B: HGF-dependent: Stimulation of Met by exogenous HGF occurs independently of Src and FAK, and does not require integrin-based cell adhesion or intact actin cytoskeleton. Addition of exogenous HGF can override the dependence of Met activation on Src and integrin-based cell adhesion. We speculate that HGF-dependent (paracrine or autocrine) activation of Met is involved primarily during early stages of breast cancer development corresponding to ductal carcinoma in situ, whereas HGF-independent Met activation may be particularly important as carcinoma cells engage integrins and invade through the surrounding stroma. F, filamentous; ECM, extracellular matrix.

treatment [Finn et al. 2007]. Inhibition of Src family kinases is therefore likely to be a useful strategy to block constitutive activation of Met and other RTKs in invasive breast and other cancer types.

In summary, our findings identify a novel role of the Src/FAK interaction network in positively regulating Met activation in an HGF-independent manner in breast epithelial cells. Furthermore, the activating effect of Src on Met requires integrin-based cell adhesion and an intact cortical cytoskeleton. In contrast, stimulation of Met by exogenous HGF can occur independently of Src. In addition, we and others have shown previously that exogenous HGF can stimulate Met activation in detached cells, indicating that integrin-based cell adhesion is not required [Qiao et al., 2000]. Thus HGF-induced Met activation likely occurs via a different mechanism than Src-induced Met activation in the absence of HGF (Fig. 9). In some carcinomas, activated Src can act co-operatively with Stat3 to stimulate HGF expression, supporting a role of Src in establishing an autocrine HGF/Met loop [Hung and Elliott, 2001; Wojcik et al., 2006]. Interestingly, these cells still require cell–matrix adhesion for full activation of Met, indicating that both ligand-dependent and ligand-independent mechanisms are involved in some carcinoma types. We speculate that ligand-dependent (paracrine or autocrine) activation of Met is involved primarily during early stages of breast cancer development corresponding to ductal carcinomas in situ, whereas ligand-independent Met activation may be particularly important as carcinoma cells engage integrin adhesion and invade through the basement membrane and surrounding stroma. Thus, targeting Src/integrin-dependent Met activation may be important in designing new drug therapies to attenuate invasiveness of breast cancer cells.

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