SHP2 Mediates the Localized Activation of Fyn Downstream of the $\alpha 6 \beta 4$ Integrin To Promote Carcinoma Invasion[▽]

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Src family kinase (SFK) activity is elevated in many cancers, and this activity correlates with aggressive tumor behavior. The $\alpha 6 \beta 4$ integrin, which is also associated with a poor prognosis in many tumor types, can stimulate SFK activation; however, the mechanism by which it does so is not known. In the current study, we provide novel mechanistic insight into how the $\alpha 6 \beta 4$ integrin selectively activates the Src family member Fyn in response to receptor engagement. Both catalytic and noncatalytic functions of SHP2 are required for Fyn activation by $\alpha 6 \beta 4$. Specifically, the tyrosine phosphatase SHP2 is recruited to $\alpha 6 \beta 4$ and its catalytic activity is stimulated through a specific interaction of its N-terminal SH2 domain with pY1494 in the $\beta 4$ subunit. Fyn is recruited to the $\alpha 6 \beta 4$ /SHP2 complex through an interaction with phospho-Y580 in the C terminus of SHP2. In addition to activating Fyn, this interaction with Y580-SHP2 localizes Fyn to sites of receptor engagement, which is required for $\alpha 6 \beta 4$ -dependent invasion. Of significance for tumor progression, phosphorylation of Y580-SHP2 and SFK activation are increased in orthotopic human breast tumors that express $\alpha 6 \beta 4$ and activation of this pathway is dependent upon Y1494.

Expression of the $\alpha 6 \beta 4$ integrin, a laminin receptor, is associated with poor patient prognosis and reduced survival in many human cancers (32). For this reason, there is considerable interest in understanding how this integrin is regulated and how it functions to promote tumor progression. In normal tissues, the $\alpha 6 \beta 4$ integrin plays a major role in maintaining the integrity of epithelia by binding to laminins in the basement membrane and regulating the assembly of hemidesmosomes on the basal epithelial cell surface (7, 17). In pathophysiological conditions such as wound healing and cancer, the stable adhesive interactions of the $\alpha 6 \beta 4$ receptor are disrupted by phosphorylation of the $\beta 4$ cytoplasmic domain, converting $\alpha 6 \beta 4$ to a signaling-competent receptor that promotes dynamic adhesion and invasion (18). Phosphorylation of the $\beta 4$ subunit cytoplasmic domain on serine residues contributes to the dynamic adhesive functions of the receptor by disrupting interactions with hemidesmosomal proteins that regulate stable adhesion (33, 37). Phosphorylation of the $\beta 4$ cytoplasmic domain on tyrosine residues may also contribute to the regulation of hemidesmosomes, but it is likely that the major contribution of tyrosyl phosphorylation is to mediate interactions that stimulate downstream signaling from the receptor (22).

In transformed cells, engagement of the $\alpha 6 \beta 4$ integrin stimulates the activation of several signaling molecules, including phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinases (MAPK), NF κ B, and Src family kinases (SFKs) (10, 12, 21, 40). In previous studies, we identified Y1494 in the $\beta 4$ subunit cytoplasmic domain as an important mediator of $\alpha 6 \beta 4$ -dependent signaling by demonstrating that mutation of Y1494 inhibits the ability of $\alpha 6 \beta 4$ to stimulate PI3K, MAPK,

and SFK activation (10, 39). Restoration of both PI3K and SFK signaling, but not MAPK signaling, rescues invasion in tumor cells expressing Y1494F- $\beta 4$, indicating that PI3K and SFK signaling pathways cooperate downstream of Y1494 to promote $\alpha 6 \beta 4$ -dependent invasion (10). Y1494 is localized within an immunoreceptor tyrosine-based inhibition motif (ITIM), a canonical binding site for Src-homology-2 (SH2) domain-containing protein-tyrosine phosphatase 1 (SHP1) and SHP2 (44). Examination of a chimeric receptor containing the extracellular domain of TrkB and the transmembrane and cytoplasmic domains of the $\beta 4$ subunit demonstrated that SHP2 binds to and is activated by sequences in the $\beta 4$ cytoplasmic domain in response to dimerization (23). Moreover, Y1494 is one of three tyrosine residues, along with Y1257 and Y1440, that mediate the interaction of SHP2 with the $\beta 4$ subunit cytoplasmic domain in response to c-Met signaling (6). Importantly, SHP2 is essential for the activation of SFKs both by the chimeric TrkB/ $\beta 4$ receptor and when the $\beta 4$ subunit functions as a signaling adaptor for c-Met (6, 23). However, the mechanism by which SHP2 activates SFKs in response to $\alpha 6 \beta 4$ engagement has not been established.

Elevated SFK activity correlates strongly with breast cancer invasion and metastasis, and these kinases are frequently activated in human cancers (15). Given the parallels between $\alpha 6 \beta 4$ expression and SFK activation in cancer, investigation of how $\alpha 6 \beta 4$ contributes to the activation of this invasion-promoting pathway is warranted. In the current study, we sought to elucidate the mechanism by which engagement of $\alpha 6 \beta 4$ activates SFKs and the significance of the $\beta 4$ /SHP2/SFK signaling axis for tumor progression. Our results reveal a novel mechanism for SHP2-dependent activation of the SFK family member Fyn which involves Y580 in the C terminus of SHP2.

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MATERIALS AND METHODS

Cell lines, antibodies (Abs), and reagents. MDA-MB-435 cells expressing wild-type (WT) and mutant $\beta 4$ subunits were generated and maintained as

described previously (39, 40). MDA-MB-231 human breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository (Georgetown University) and maintained in RPMI medium containing 10% fetal calf serum.

The following Abs were used: phospho-Y542 SHP2 (catalog no. 3751) and phospho-Y580 SHP2 (catalog no. 3754) from Cell Signaling; SHP2 (catalog no. sc280), SFK (catalog no. sc8056), Src (catalog no. sc-19), Fyn (catalog no. sc-16), and Yes (catalog no. sc-14) from Santa Cruz Biotechnology, Inc.; phospho-Y418 Src (catalog no. 44660G) from Invitrogen; $\beta 4$ subunit (439-9B from R. Falcioni [Regina Elena Cancer Institute], and UM-A9 from Ancell, and cytoplasmic domain polyclonal antiserum from A. Mercurio [UMass Medical School]); pY1494- $\beta 4$ from ECM Biosciences (catalog no. IP1281); $\alpha 6$ subunit (2B7) from A. Mercurio; hemagglutinin (HA; catalog no. 11867423001) from Roche; actin (catalog no. A2066) from Sigma; and anti-rat (catalog no. 112-005-003) and anti-mouse (catalog no. 115-005-003) IgGs from Jackson ImmunoResearch.

Calpeptin (catalog no. 0334 0051), calpastatin peptide (catalog no. 208902), ALLN (catalog no. 208719), and PP2 (catalog no. 529573) were obtained from Calbiochem. Matrigel (catalog no. 356237) was obtained from BD Biosciences, and murine laminin 1 was from Trevigen (catalog no. 3400-010-01) or Stemgent (catalog no. 06-0002). WT and mutant SHP2 constructs and SHP2 short hairpin RNA (shRNA) in the pSUPER retroviral vector were gifts from B. Neel (Ontario Cancer Institute, Toronto, Canada). SHP2 SH2-GST fusion constructs were gifts from Eugene Chin (Brown University) (47). Human Fyn small interfering RNA (siRNA) was obtained from Qiagen (catalog no. SI02659545).

Integrin clustering. Cells were serum starved overnight in medium containing 0.1% bovine serum albumin (BSA). Cells were trypsinized and washed before being resuspended at a concentration of 2×10^6 /ml and incubated for 30 min with or without integrin-specific Abs (2 μ g/ml) in medium containing 0.1% BSA. The cells were washed once and added to plates which had been coated overnight with either anti-mouse or anti-rat IgG (100 μ g/10-cm plate), laminin (550 μ g/10-cm plate) or BSA (1%). Chemical inhibitors were added to the cells for 10 min of incubation prior to plating of the cells in the coated plates. After incubation at 37°C for 30 min, the cells were washed once with PBS and lysed in 20 mM Tris buffer, pH 7.4, containing 10% glycerol, 136 mM NaCl, 10% NP-40, 5 mM EDTA, 1 mM sodium orthovanadate (Na_3VO_4), and complete protease inhibitor cocktail (Roche) (lysis buffer A).

Immunoprecipitation and immunoblotting. Cell extracts containing equivalent amounts of total protein were incubated for 3 h or overnight at 4°C with Abs. Either protein A- or protein G-conjugated Sepharose beads were added, and the mixture was incubated for an additional 1 to 2 h. Immune complexes were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes for immunoblotting as described previously (10).

SHP2 *in vitro* tyrosine phosphatase assay. SHP2 phosphatase activity was measured *in vitro* using PTP Assay Kit 1 from Upstate Biotechnology. Cells were extracted in lysis buffer A without phosphatase inhibitors. Cell extracts containing equivalent amounts of total protein were incubated overnight with SHP2-specific Abs and protein G-Sepharose beads. The beads were washed four times with 10 mM Tris-HCl, pH 7.4, and then resuspended in the same buffer with a tyrosine phosphopeptide (0.1 mM) and incubated with gentle agitation for 1 h at 37°C. The reaction was terminated by the addition of malachite green. SHP2 phosphatase activity was measured in a microtiter plate reader at 620 nm by following the manufacturer's instructions.

Two-dimensional (2D) invasion and adhesion assays. Matrigel invasion assays were performed using 6.5-mm Transwell chambers (8- μ m pore size; Costar) (10). Matrigel, purified from the Englebreth-Holm-Swarm tumor, was diluted in cold distilled water, added to the Transwells (5 μ g/well), and dried in a sterile hood. The Matrigel was then reconstituted with medium for 1 h at 37°C before the addition of cells. Cells (0.5×10^5) were resuspended in serum-free Dulbecco's modified Eagle's medium containing 0.1% BSA and added to each well. Conditioned NIH 3T3 medium was added to the bottom wells of the chambers. After 5 h, the cells that had invaded to the lower surface of the filters were fixed in methanol for 10 min. The fixed membranes were mounted on glass slides using Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Invasion was quantified by counting the stained nuclei in five independent fields in each Transwell.

Laminin adhesion assays were performed as described previously (39). Briefly, multiwell tissue culture plates (11.3-mm diameter) were coated overnight at 4°C with 0.2 ml of phosphate-buffered saline (PBS) containing murine laminin 1 (20 μ g/ml). The wells were then washed with PBS and blocked with RPMI medium containing 0.1% BSA. Cells (10^5) were resuspended in blocking buffer and added to the protein-coated wells. After a 60-min incubation at 37°C, the wells were washed three times, fixed for 15 min with methanol, and stained with a 0.2% solution of crystal violet in 2% ethanol. After washing, the crystal violet stain was

solubilized with a 1% solution of SDS and adhesion was quantitated by measuring the absorbance at 595 nm.

3D Matrigel invasion assay. A base layer of Matrigel (200 μ l/well) was overlaid in duplicate wells of a 24-well dish with 1.0×10^4 cells suspended in 300 μ l of a 2:1 mixture of PBS and Matrigel. The Matrigel was overlaid with complete serum-containing medium (0.5 ml/well), which was changed every 3 days. Images were captured with SPOT image analysis software (Molecular Diagnostics).

Tumor extraction. Frozen tumors were homogenized at 4°C in T-PER tissue protein extraction reagent (Pierce Biotechnology, Inc.) containing 1 mM sodium orthovanadate, 10 mM NaF, and protease inhibitors (Complete mini; Roche Applied Science).

Statistics. All data are represented as means ($\pm$ standard error or standard deviation). All statistical analyses were performed using the unpaired Student *t* test.

RESULTS

Recruitment and activation of SHP2 by the $\alpha 6 \beta 4$ integrin.

We sought to determine if Y1494 in the $\beta 4$ subunit cytoplasmic domain plays a role in the recruitment of SHP2 to the $\alpha 6 \beta 4$ integrin in response to receptor ligation and if this binding stimulates SHP2 phosphatase activity. Ligation of $\alpha 6 \beta 4$ with either $\alpha 6$ -specific Abs or laminin 1-stimulated phosphorylation of Y1494 (Fig. 1A). To investigate if phosphorylation of Y1494 is required for the binding of SHP2 to the $\beta 4$ subunit, biotinylated peptides corresponding to 14 amino acids of the $\beta 4$ cytoplasmic domain surrounding Y1494 and the ITIM binding motif were synthesized (Fig. 1B; ITIM underlined). Phosphotyrosyl was incorporated into the Y1494 site of one of the peptides to determine the importance of tyrosine phosphorylation of Y1494 for binding. These peptides were used to pull down SHP2 from extracts of MDA-MB-231 human breast carcinoma cells, which express the $\alpha 6 \beta 4$ integrin and SHP2 endogenously. SHP2 was precipitated from the cell extracts by the phosphorylated $\beta 4$ peptide but not by the nonphosphorylated peptide (Fig. 1B). To demonstrate an endogenous interaction of SHP2 with the $\alpha 6 \beta 4$ receptor, cell extracts were immunoprecipitated with either nonspecific IgG or $\beta 4$ -specific Abs and immunoblotted for SHP2. Adhesion to laminin stimulated the association of SHP2 with $\alpha 6 \beta 4$ (Fig. 1C).

SHP2 contains two SH2 domains in the N terminus of the protein that mediate its interactions with phosphotyrosyl residues to recruit the phosphatase to signaling complexes and activate the catalytic activity (26). To determine how SHP2 interacts with the $\beta 4$ subunit, we examined the ability of SHP2-glutathione *S*-transferase (GST) fusion proteins containing both SH2 domains (GST-N-C-SH2), the N-terminal SH2 domain (GST-N-SH2), or the C-terminal SH2 domain (GST-C-SH2) to pull down the $\beta 4$ subunit from extracts of MDA-MB-435 cells that were transfected with the WT $\beta 4$ subunit (WT- $\beta 4$). To increase the phosphorylation of the $\beta 4$ cytoplasmic domain, the cells were treated briefly with a sodium orthovanadate-hydrogen peroxidase ($\text{Na}_3\text{VO}_4\text{-H}_2\text{O}_2$) mixture. Phosphorylation of the $\beta 4$ subunit is markedly increased in the presence of $\text{Na}_3\text{VO}_4\text{-H}_2\text{O}_2$ (39). All three SHP2/SH2-GST fusion proteins pulled down the full-length WT- $\beta 4$ subunit from extracts of treated cells (Fig. 1D), indicating that both SH2 domains of SHP2 engage the $\beta 4$ subunit. To identify the specific tyrosine residues in the $\beta 4$ subunit that interact with SHP2, we next assessed the ability of the SHP2-GST fusion proteins to pull down $\beta 4$ subunits containing point mutations to phenylalanine at Y1494 (Y1494F- $\beta 4$) or Y1440

FIG. 1. pY1494 in the $\beta 4$ integrin subunit is required for SHP2 activation. (A) MDA-MB-435 cells transfected with either WT- $\beta 4$ or Y1494F- $\beta 4$ were incubated with (+) or without (–) $\alpha 6$ -specific Abs and allowed to adhere to anti-mouse IgG- or laminin (LN1)-coated plates. Aliquots of cell lysates were immunoblotted with Abs specific for phospho-Y1494 of the $\beta 4$ subunit. The phosphoimmunoblot was stripped and reprobed for total $\beta 4$ expression levels. (B) MDA-MB-231 cell lysates were incubated with biotinylated peptides bound to streptavidin-coated beads, and bound proteins were immunoblotted with SHP2-specific Abs. (C) MDA-MB-435/WT- $\beta 4$ cells were allowed to adhere to BSA (–) or laminin 1 (+)-coated plates for 30 min. Aliquots of cell lysates were immunoprecipitated (IP) with nonspecific IgG or $\beta 4$ -specific Abs ($\beta 4$) and immunoblotted with Abs specific for SHP2 and $\beta 4$. Total cell lysates were also immunoblotted with SHP2- and $\beta 4$ -specific Abs (input). (D) SHP2 GST fusion proteins containing both SH2 domains (GST-N-C-SH2), the N-terminal SH2 domain (GST-N-SH2), or the C-terminal SH2 domain (GST-C-SH2) and GST alone were affinity purified with glutathione-Sepharose beads, and their expression was confirmed by GST immunoblotting (left). Cell extracts from MDA-MB-435/WT- $\beta 4$, MDA-MB-435/Y1494F- $\beta 4$, and MDA-MB-435/Y1440F- $\beta 4$ transfectants left untreated or treated with Na₃VO₄-H₂O₂ to increase tyrosine phosphorylation were incubated with the SHP2-GST fusion proteins and then immunoblotted with a $\beta 4$ -specific antiserum (right). WT- $\beta 4$, MDA-MB-435 cells transfected with the WT $\beta 4$ subunit; Y1494F- $\beta 4$, MDA-MB-435 cells transfected with the Y1494F mutant $\beta 4$ subunit; Y1440F- $\beta 4$, MDA-MB-435 cells transfected with the Y1440F mutant $\beta 4$ subunit. (E) MDA-MB-435/WT- $\beta 4$, MDA-MB-435/Y1494F- $\beta 4$, MDA-MB-435-Y1257F, Y1494F- $\beta 4$, and MDA-MB-435/Y1440F- $\beta 4$ transfectants were incubated with or without $\alpha 6$ -specific Abs and allowed to adhere to either anti-mouse IgG- or laminin 1-coated plates, respectively, for 30 min. Aliquots of cell lysates were immunoprecipitated with $\beta 4$ -specific Abs and immunoblotted with Abs specific for SHP2 and $\beta 4$. Total cell lysates were also immunoblotted with SHP2 (input)-, Y1494F- $\beta 4$ -, or $\beta 4$ -specific Abs. (F and G) MDA-MB-435 cells transfected with the vector alone (Mock), WT- $\beta 4$, or Y1494F- $\beta 4$ and MDA-MB-231 cells stably expressing either empty vector (EV) or dominant negative SHP2 (DN-SHP2) were incubated with or without $\beta 4$ -specific Abs and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with SHP2-specific Ab-conjugated agarose beads. The immune complexes were incubated with phosphotyrosyl peptides to assay phosphatase activity. One-tenth of the total immune complex was immunoblotted to determine the input. The data shown represent the mean ($\pm$ the standard deviation) of three independent experiments. *, $P \leq 0.004$; **, $P \leq 0.005$. The values to the left of the blots are molecular sizes in kilodaltons.

FIG. 2. SHP2 phosphatase activity is required for $\alpha 6\beta 4$ -dependent SFK activation. (A) MDA-MB-231 cells stably expressing empty vector (EV), HA-tagged dominant negative SHP2 (DN), or SHP2-shRNA (KD) were maintained in suspension or incubated with $\beta 4$ -specific Abs and allowed to adhere to anti-mouse IgG-coated plates for 30 min. Aliquots of cell lysates were immunoblotted with Abs specific for pY418-SFK, SFK, SHP2, or actin. The data shown in the graph represent the mean ($\pm$ the standard deviation) of three independent experiments that were quantified by densitometry. *, $P \leq 0.04$; **, $P \leq 0.01$. (B and C) MDA-MB-435 cells transfected with WT- $\beta 4$, Y1494F- $\beta 4$, Y1257F, Y1494F- $\beta 4$, or Y1440F- $\beta 4$ were incubated with or without $\alpha 6$ -specific Abs and allowed to adhere to either anti-mouse IgG (B)- or laminin (C)-coated plates. Aliquots of cell lysates were immunoblotted with Abs specific for pY418-SFK. The pY418-SFK immunoblots were stripped and reprobed for total SFK expression. The values to the left of the blots are molecular sizes in kilodaltons.

(Y1440F- $\beta 4$). These tyrosines had been previously implicated in the interaction of SHP2 with $\alpha 6\beta 4$ in response to c-Met stimulation using far-Western analysis (6). Mutation of Y1494 significantly reduced the binding of only the GST-N-SH2 fusion protein to the $\beta 4$ subunit, whereas mutation of Y1440 significantly reduced the binding of both individual SH2 domains (Fig. 1D). The diminished binding of the N-SH2 domain to Y1440F- $\beta 4$ likely reflects the dependence of Y1494 phosphorylation on an intact Y1440 (Fig. 1E). Although the double N-C-SH2 domains were capable of pulling down the WT and mutant $\beta 4$ subunits, the relative level of binding of the mutant subunits was significantly diminished compared with WT binding when total $\beta 4$ expression levels were normalized (data not shown), suggesting that both of these tyrosine residues are required for the stable recruitment of SHP2 to the $\beta 4$ subunit. In support of this requirement, mutation of either Y1494 or Y1440 alone inhibited the ability of SHP2 to coimmunoprecipitate with $\alpha 6\beta 4$ after adhesion to laminin (Fig. 1E).

Structural analysis of SHP2 has revealed that the N-SH2 domain forms contacts with the catalytic domain and, in doing so, blocks the access of substrates to the active site (4). This inactive state switches to an active state upon the binding of a phosphopeptide to the N-SH2 domain (41). To determine if ligation of $\alpha 6\beta 4$ promotes SHP2 activity and to investigate the contribution of Y1494, which binds to the N-SH2 domain, to this activation, SHP2 *in vitro* phosphatase assays were performed after $\alpha 6\beta 4$ ligation. Engagement of $\alpha 6\beta 4$ increased SHP2 phosphatase activity by approximately 65% in MDA-MB-435/WT- $\beta 4$ cells but had no effect on mock-transfected cells that do not express the $\alpha 6\beta 4$ receptor (Fig. 1F). A 2-fold

induction of phosphatase activity was also observed upon ligation of endogenous $\alpha 6\beta 4$ in MDA-MB-231 cells, and this increase was inhibited by the expression of a catalytically inactive, substrate-trapping SHP2 mutant (C459S-SHP2) that functions in a dominant negative manner (Fig. 1G) (16). Importantly, mutation of Y1494 inhibited the increase in SHP2 phosphatase activity in response to receptor ligation (Fig. 1F). We conclude from these studies, taken together with our GST-binding data, that Y1440 is the primary binding site in the $\beta 4$ subunit for the C-SH2 domain of SHP2 and Y1494 is the primary binding site in the $\beta 4$ subunit for the N-SH2 domain of SHP2, and both domains are required for stable interaction. Furthermore, recruitment of the N-SH2 domain to Y1494 is required for the stimulation of SHP2 catalytic activity in response to $\alpha 6\beta 4$ engagement.

SHP2 phosphatase activity is required for SFK activation. Previous work from our own lab and others has implicated SHP2 in the activation of SFKs downstream of the $\alpha 6\beta 4$ integrin (6, 23). However, the mechanism by which SHP2 stimulates this activation in response to $\alpha 6\beta 4$ ligation has not been elucidated. To investigate if SHP2 phosphatase activity is required for SFK activation, MDA-MB-231 cells that stably express the catalytically inactive SHP2 mutant (DN-SHP2) were assayed for their activation of SFKs in response to $\alpha 6\beta 4$ ligation, as measured by autophosphorylation of the activation loop (Y418 in human c-Src). The pY418-Src Ab recognizes the SFK family members Src, Fyn, and Yes. The exogenously expressed SHP2 mutant was expressed at levels approximately equivalent to those of endogenous SHP2, as evidenced by the more slowly migrating band of the HA-tagged DN-SHP2 mu-

FIG. 3. SFKs phosphorylate Y542, but not Y580, in the C-terminal tail of SHP2. Cells were incubated with or without integrin-specific Abs and allowed to adhere to anti-mouse IgG-coated plates or laminin 1-coated plates. Aliquots of cell lysates were immunoblotted with Abs specific for the indicated phosphoproteins. The phosphoimmunoblots were stripped and reprobed for total protein expression levels. (A) MDA-MB-231 cells were ligated with (+) or without (–) β 4-specific Abs. (B) MDA-MB-435/WT- β 4 cells were ligated with (+) or without (–) α 6-specific Abs. (C) MDA-MB-435/WT- β 4 cells were allowed to adhere to laminin 1-coated plates for the indicated time periods. (D) MDA-MB-435 cells that were transfected with WT- β 4 or the empty vector (Mock) were ligated with α 6-specific Abs in the absence or presence of the SHP2 inhibitor calpeptin (CP; 50 μ g/ml) or the SFK inhibitor PP2 (10 μ M). (E) MDA-MB-435/WT- β 4 cells were ligated with α 6-specific Abs in the absence or presence of calpeptin (CP; 50 μ g/ml), ALLN (50 μ M), or calpastatin peptide (CS; 5 μ M). The values to the left of the blots are molecular sizes in kilodaltons.

tant (Fig. 2A). Compared with that in cells expressing the vector alone (EV), SFK activation in response to α 6 β 4 ligation in cells expressing DN-SHP2 (DN) was significantly impaired. For comparison, SFK activation was inhibited completely when SHP2 expression was suppressed by shRNA-mediated knockdown (KD), as we had previously demonstrated in MDA-MB-435/WT- β 4 cells (23). To assess further the importance of SHP2 activation in the α 6 β 4-dependent activation of SFKs, cells expressing WT- β 4, Y1440F- β 4, and Y1494F- β 4 were evaluated for SFK activation after engagement of the α 6 β 4 receptor by clustering with α 6-specific Abs (Fig. 2B) or adhesion to laminin (Fig. 2C). Mutation of either Y1440 or Y1494, which prevents the recruitment and activation of SHP2, inhibited the ability of α 6 β 4 to promote SFK activation.

SFK activation stimulates phosphorylation of Y542, but not Y580, in the C-terminal tail of SHP2 downstream of the α 6 β 4 integrin. There are two tyrosines in the C-terminal tail of SHP2 that can regulate SHP2 catalytic activity but can also serve in a noncatalytic capacity as binding sites for intermolecular interactions (31). Although some progress has been made in recent years in understanding how phosphorylation of these tyrosines contributes to SHP2 function, the detailed molecular mechanisms by which phosphorylation of these residues impacts SHP2 catalytic activity and function remain controversial and are likely to be determined by the specific upstream stimulus (3, 20). To determine if either Y542 or Y580 is phosphorylated in response to α 6 β 4 ligation, we assessed the phosphorylation status of these tyrosine residues in two metastatic carcinoma cell lines, MDA-MB-231 and MDA-MB-435. Phosphorylation of Y542 increased in response to α 6 β 4 ligation

using either α 6- or β 4-specific Abs or its physiological ligand laminin to engage the receptor (Fig. 3A to C). In contrast, Y580 was constitutively phosphorylated in both cell lines and the phosphorylation level did not increase in response to α 6 β 4 ligation. Y564 in the C-terminal tail of SHP1, which is equivalent to Y580 in SHP2, is phosphorylated by the SFK family member Lck (19). Moreover, Src is capable of phosphorylating SHP1 in an *in vitro* kinase assay (11). Therefore, we sought to determine if SFKs participate in a feedback loop to regulate SHP2 function by phosphorylating either Y542 or Y580 in the C-terminal tail. The phosphorylation of Y542 in response to α 6 β 4 ligation was completely blocked by the SFK inhibitor PP2 in MDA-MB-435/WT- β 4 cells, but the constitutive phosphorylation of Y580 was not altered (Fig. 3D). Inhibition of SHP2 phosphatase activity by calpeptin diminished SFK activation, similar to expression of DN-SHP2 (Fig. 2A), and caused a corresponding decrease in the phosphorylation of Y542 (Fig. 3D). As controls for the specificity of calpeptin's inhibition of SHP2, calpain-specific inhibitors did not diminish the phosphorylation of Y542 (Fig. 3E).

Y580 is required for the interaction of SHP2 and Fyn. To understand the functional significance of phosphorylation of Y542 and Y580 in the C terminus of SHP2 with regard to SFK activation by the α 6 β 4 integrin, MDA-MB-231 cells that stably express HA-tagged Y542F-SHP2, Y580F-SHP2, and Y542F/Y580F-SHP2 mutant proteins were generated (Fig. 4A). All of the mutant proteins were expressed at a level similar to that of HA-tagged WT-SHP2 (Fig. 4B). As we had observed for endogenous SHP2, WT-SHP2 and the Y542F-SHP2 mutant were constitutively phosphorylated on Y580 (Fig. 4B). Moreover,

FIG. 4. Phosphorylation of Y580 in the SHP2 C terminus is required for SFK activation by the $\alpha 6\beta 4$ integrin. (A) Schematic representation of HA-tagged SHP2 Y542/Y580 mutant proteins. (B) Aliquots of extracts from MDA-MB-231 cells stably expressing the HA-tagged SHP2 constructs were immunoblotted with Abs specific for pY580-SHP2 and HA. The phosphoimmunoblot was stripped and reprobed for total SHP2 levels. (C) MDA-MB-231 cells stably expressing HA-tagged SHP2 constructs were incubated with $\beta 4$ -specific Abs and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with HA-specific Abs and immunoblotted with Abs specific for pY542-SHP2. The phosphoimmunoblot was stripped and reprobed for total SHP2 expression levels. (D) MDA-MB-231 cells stably expressing the HA-tagged SHP2 constructs were incubated with (+) or without (–) $\beta 4$ -specific Abs and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoblotted with Abs specific for pY418-SFK or actin. The phosphoimmunoblot was stripped and reprobed for total SFK expression levels. The data shown in the graph represent the mean ($\pm$ the standard deviation) of three independent experiments that were quantified by densitometry. *, $P \leq 0.03$. (E) MDA-MB-231 cells stably expressing HA-tagged SHP2 constructs were incubated with $\beta 4$ -specific Abs and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with HA-specific Abs, and the immune complexes were incubated with phosphotyrosyl peptides to assay phosphatase activity. One-tenth of the total immune complex was immunoblotted to determine the input. The data shown represent the mean ($\pm$ the standard deviation) of three independent experiments. *, $P \leq 0.04$; **, $P \leq 0.005$. The values to the left of the blots are molecular sizes in kilodaltons.

ligation of $\alpha 6\beta 4$ stimulated the phosphorylation of Y542 in exogenously expressed WT-SHP2 and in the Y580F-SHP2 mutant (Fig. 4C). However, the level of Y542 phosphorylation was significantly diminished when Y580 was mutated, suggesting that phosphorylation of Y580 contributes to the SFK-dependent phosphorylation of Y542 (Fig. 4C).

Next we evaluated the contribution of the SHP2 C-terminal tyrosines to $\alpha 6\beta 4$ -dependent SFK activation. Mutation of Y542 resulted in a modest reduction in activation in response to $\alpha 6\beta 4$ ligation (Fig. 4D). In contrast, mutation of Y580 either alone or in combination with Y542 significantly diminished SFK activation (Fig. 4D). To determine if SHP2 phosphatase activity correlates with SFK activation, *in vitro* phosphatase

assays were performed after ligation of $\alpha 6\beta 4$. Mutation of Y542 and Y580 individually reduced the phosphatase activity of SHP2, whereas the activity of the Y542F/Y580F double-mutant protein was equivalent to that observed for WT-SHP2 (Fig. 4E). This finding mimics a previous report that deletion of the SHP1 C-terminal tail activates the catalytic activity of the phosphatase (29). The inability of the double-mutant protein, which retains phosphatase activity, to activate SFKs suggests that Y580 may contribute to $\alpha 6\beta 4$ -dependent SFK activation by a mechanism that is independent of its regulation of SHP2 catalytic activity.

Y580 in SHP2 is localized within a binding motif that is recognized by the SH2 domains of several SFK family mem-

FIG. 5. Y580 in the C terminus of SHP2 is required for the interaction of SHP2 and Fyn. (A) MDA-MB-231 cells were incubated with or without $\beta 4$ -specific Abs and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated (IP) with nonspecific IgG or SFK Abs and immunoblotted with Abs specific for SHP2. The immunoblot was stripped and reprobed for total SFK expression levels. Total cell lysates were also immunoblotted with SHP2-specific Abs (SHP2 input). (B) Aliquots of cell lysates were immunoblotted with Abs specific for Src, Fyn, Yes, and actin. 231, MDA-MB-231 cells; 435- $\beta 4$, MDA-MB-435 cells transfected with WT- $\beta 4$. (C) MDA-MB-231 and MDA-MB-435/WT- $\beta 4$ cells were incubated with integrin-specific Abs and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with either nonspecific IgG or Abs that specifically recognize Fyn or Src and immunoblotted with Abs specific for SHP2. The immunoblot was stripped and reprobed for total Fyn or Src expression levels, respectively. Total cell lysates were also immunoblotted with SHP2-specific Abs (SHP2 input). (D) MDA-MB-231 cells were transfected with siRNA specific for either luciferase (Luc) or human Fyn for 48 h. Cells were incubated with or without $\alpha 6$ -specific Abs and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoblotted with Abs specific for Fyn, pY542-SHP2, or actin. The phosphoblot was stripped and reprobed for total SHP2 expression. (E) MDA-MB-231 cells stably expressing HA-tagged SHP2 constructs were incubated with $\beta 4$ -specific Abs and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with Abs that recognize either HA or Fyn and immunoblotted with Abs that recognize either Fyn (HA-IP) or SHP2 (Fyn-IP). The immunoblots were stripped and reprobed for total HA or Fyn expression levels, respectively. Total cell lysates were also immunoblotted with SHP2 and Fyn Abs (Input). The values to the left of the blots are molecular sizes in kilodaltons.

bers (Scansite). Therefore, we sought to determine if SHP2 and SFKs physically interact with each other and if this interaction is regulated by $\alpha 6\beta 4$ engagement. In MDA-MB-231 cells, pan-SFK Abs coimmunoprecipitated SHP2 in the absence of $\alpha 6\beta 4$ ligation and the SFK-SHP2 interaction increased upon ligation of the receptor (Fig. 5A). MDA-MB-231 cells express only the SFK family member Fyn, whereas MDA-MB-435 cells express both Src and Fyn. Neither cell line expresses the SFK family member Yes (Fig. 5B). To determine if there is specificity in the binding of SFKs to SHP2, Abs that selectively recognize individual family members were used for the immunoprecipitations. SHP2 coimmunoprecipitated with Fyn from both cell lines, but no interaction with Src was observed in the MDA-MB-435/WT- $\beta 4$ cells, indicating that Fyn, but not Src, is recruited to SHP2 downstream of $\alpha 6\beta 4$ (Fig.

5C). Signaling through Fyn is demonstrated further by the fact that suppression of Fyn expression by siRNA diminished phosphorylation of Y542-SHP2 in response to $\alpha 6\beta 4$ engagement (Fig. 5D). To investigate further the contribution of Y542 and Y580 to the interaction of SHP2 with Fyn, HA-specific Abs were used to immunoprecipitate exogenously expressed SHP2 proteins after ligation of $\alpha 6\beta 4$. Fyn coimmunoprecipitated with WT-SHP2 and also with the Y542F-SHP2 mutant. However, mutation of Y580 prevented the interaction of SHP2 with Fyn (Fig. 5E). The reverse immunoprecipitation using SFK Abs revealed a similar requirement for an intact Y580 to pull down SHP2 (Fig. 5E). In contrast, a GST fusion protein containing the tandem SH2 domains of SHP2 failed to pull down Fyn, indicating that the interaction of Fyn with SHP2 is independent of these domains (data not shown).

FIG. 6. A positive role for the $\alpha 6\beta 4$ -SHP2-Fyn pathway in tumor cell invasion. (A) MDA-MB-435 cells transfected with WT- $\beta 4$, Y1494F- $\beta 4$, Y1257F/Y1494F- $\beta 4$, or Y1440F- $\beta 4$ were assayed for the ability to invade Matrigel using a Transwell assay chamber. The data shown represent the mean ($\pm$ the standard deviation) of five independent invasion assays performed in duplicate. (B) MDA-MB-231 cells transfected with either the empty vector or the indicated HA-tagged SHP2 constructs were assayed for the ability to invade Matrigel using a Transwell assay chamber. The data shown represent the mean ($\pm$ the standard deviation) of three independent invasion assays performed in duplicate. *, $P \leq 0.02$; **, $P \leq 0.008$. (C) MDA-MB-231 cells transfected with either the empty vector or the indicated HA-tagged SHP2 constructs were allowed to adhere to laminin 1-coated wells for 1 h at 37°C. After washing, cells were fixed, stained with crystal violet, and quantified by measuring absorbance at 595 nm. The data shown represent the mean ($\pm$ the standard deviation) of three independent assays performed in triplicate. (D) Representative images captured at $\times 20$ magnification of MDA-MB-231 cells transfected with the indicated HA-tagged SHP2 constructs or incubated in the presence of PP2 and grown for 10 days in 3D Matrigel culture.

A positive role for the $\alpha 6\beta 4$ -SHP2-Fyn pathway in tumor cell invasion. The $\alpha 6\beta 4$ integrin promotes tumor cell invasion, and SFK activation is required for this $\alpha 6\beta 4$ -dependent function (10, 40). Mutation of either Y1440 or Y1494 in the $\beta 4$ subunit cytoplasmic domain inhibits SHP2 recruitment and activation (Fig. 1) and also impairs the ability of $\alpha 6\beta 4$ to promote carcinoma invasion (Fig. 6A). To evaluate the overall importance of SHP2 for carcinoma invasion, MDA-MB-231 cells expressing WT-SHP2 and SHP2 mutant proteins were assayed for their invasive potential using Transwell invasion chambers. Expression of WT-SHP2 increased invasion, and expression of DN-SHP2 decreased invasion, compared with

that in cells expressing the empty vector (Fig. 6B). Expression of the Y542F-SHP2, Y580F-SHP2, and Y542F/Y580F-SHP2 mutant proteins also increased invasion above the level observed in the vector control cells. However, the invasion was significantly lower in all three of the mutant cell lines compared with that in WT-SHP2-expressing cells. Previous studies have implicated SHP2 in the regulation of cell adhesion and spreading, which could influence invasive potential (28). To determine if changes in cell adhesion could explain the differences in cell invasion that were observed for mutant SHP2-expressing cells, the cells were also assayed for their adhesion to laminin 1 substrates. As shown

FIG. 7. Phosphorylation of Y580-SHP2 and Y418-SFK in human orthotopic breast tumors. (A) Aliquots of extracts from mock-treated cells or WT- β 4- and Y1494F- β 4-derived tumors were immunoblotted with Abs specific for pY580-SHP2 and pY418-SFK. The immunoblots were stripped and reprobed for total SHP2 and SFK expression levels. (B) The data shown in the graphs represent the mean levels of pY580-SHP2/SHP2 and pY418-SFK/SFK that were quantified by densitometry for each tumor type ($n = 6$ for each tumor type). *, $P \leq 0.003$; **, $P \leq 0.0004$; ***, $P \leq 0.02$. The values to the left of the blots are molecular sizes in kilodaltons.

in Fig. 6C, all of the cell lines adhered to laminin at equivalent levels.

To investigate the contribution of SHP2 to invasion in a 3D assay that more accurately reflects the tumor microenvironment, cells were embedded within a Matrigel matrix. When grown in a 3D matrix, noninvasive cells form round, compact colonies whereas invasive cells exhibit a stellate, invasive morphology. Cells expressing WT-SHP2 formed very diffuse invasive colonies, as we had observed previously for parental MDA-MB-231 and MDA-MB-435/WT- β 4 cells (Fig. 6D) (10). In contrast, cells expressing Y542F-SHP2, Y580F-SHP2, and Y542F/Y580F-SHP2 formed progressively less invasive colonies, with the double-mutant Y542F/Y580F-SHP2-expressing cells being the least invasive of the SHP2 C-terminal tyrosine mutant cell lines. Moreover, expression of DN-SHP2 completely inhibited invasion in the 3D Matrigel matrix and the colonies formed by cells expressing this catalytically inactive mutant SHP2 protein were similar in morphology to parental MDA-MB-231 cells that were grown in the presence of the SFK inhibitor PP2 (Fig. 6D) and to MDA-MB-435/Y1494F- β 4 cells (10). Taken together, the 2D and 3D invasion assays demonstrate that the abilities of α 6 β 4 to recruit and activate SHP2 and to activate Fyn are essential for the α 6 β 4 integrin to optimally promote invasion.

α 6 β 4-dependent phosphorylation of Y580-SHP2 and Y418-SFK in orthotopic human tumors. Our *in vitro* studies implicate Y580-SHP2 as an important regulator of Fyn activation and carcinoma invasion downstream of the α 6 β 4 integrin. To determine if this α 6 β 4/SHP2/Fyn signaling pathway is active *in vivo*, we assessed the phosphorylation status of Y580-SHP2

FIG. 8. Schematic of α 6 β 4-SHP2-Fyn signaling. In the inactive state, the α 6 β 4 integrin and Fyn are localized in proximity in the membrane through their palmitoylation. However, neither Fyn nor SHP2 is active because of intramolecular inhibitory interactions. Upon engagement of the α 6 β 4 integrin with its ligand laminin (active state), the β 4 cytoplasmic domain is phosphorylated, which recruits SHP2 through an interaction of its C-SH2 domain with pY1440 and activates SHP2 catalytic activity through the interaction of its N-SH2 domain with pY1494. Fyn is recruited to the complex and activated upon the binding of its SH2 domain to pY580 in the SHP2 C-terminal domain. Although SHP2 phosphatase activity is required for Fyn activation, the specific SHP2 substrates involved are unknown. The localized activation of this α 6 β 4/SHP2/FYN signaling pathway promotes carcinoma invasion. *, phosphorylation event.

and Y418-SFK in tumors that were generated using MDA-MB-435 cells expressing empty vector, WT- β 4, or Y1494F- β 4 (10). As we have previously reported, phosphorylation of Y418-SFK is enhanced significantly in tumors that express α 6 β 4 and this activation is dependent upon Y1494, which is required for SHP2 recruitment and activation (Fig. 7) (10). Y580-SHP2 phosphorylation correlates with pY418-SFK, as it is also enhanced by expression of α 6 β 4 and suppressed by mutation of Y1494 (Fig. 7). Taken together, these results support a potential role for this signaling pathway in the more aggressive behavior of tumors that express α 6 β 4.

DISCUSSION

In this study, we elucidate a novel mechanism by which SHP2 mediates the selective activation of Fyn by the α 6 β 4 integrin and demonstrate the importance of this α 6 β 4/SHP2/Fyn signaling pathway for carcinoma invasion (Fig. 8). Engagement of the α 6 β 4 integrin promotes the interaction of the N- and C-terminal SH2 domains of SHP2 with Y1494 and Y1440, respectively, in the β 4 subunit cytoplasmic domain, which stimulates the catalytic activity of SHP2. Fyn, but not Src, is recruited to SHP2 through phospho-Y580 in the C terminus of SHP2, and this interaction is necessary for the activation of Fyn downstream of α 6 β 4. Upon activation, Fyn phosphorylates SHP2 on Y542, creating a positive feedback loop that contributes to sustained SHP2 signaling. Both α 6 β 4 mutants that cannot recruit and activate SHP2 and SHP2 mutants that cannot recruit and activate Fyn have diminished abilities to promote breast carcinoma invasion. *In vivo*, pY580-SHP2 and pY418-SFK levels are increased in tumors that express the α 6 β 4 integrin, and this activation is dependent upon Y1494.

Taken together, our results reveal how the $\alpha 6 \beta 4$ integrin localizes Fyn activation to promote breast carcinoma invasion and identify pY580-SHP2 and pY1494- $\beta 4$ as potential indicators of invasive potential.

We originally identified Y1494 as a potential regulator of signaling from the $\alpha 6 \beta 4$ integrin based upon its localization within a consensus-binding motif for the SH2 domain containing tyrosine phosphatases SHP1 and SHP2 (39, 44). Although it has been known for some time that mutation of Y1494 significantly impairs $\alpha 6 \beta 4$ -dependent invasion, the mechanism by which this tyrosine residue controls invasion had not been clearly elucidated (39). Mutation of Y1494 diminishes the activation of both PI3K and SFK signaling pathways, which cooperate to promote invasion by $\alpha 6 \beta 4$ (10, 39). Activation of PI3K is mediated through the insulin receptor substrate adaptor proteins or through cooperation with growth factor receptors (18, 39). In a previous study that investigated the function of the $\beta 4$ subunit as a signaling adapter for the c-Met receptor, Y1494 was identified as one of three tyrosine residues that were required for SHP2 interaction with the $\beta 4$ subunit in response to hepatocyte growth factor stimulation to promote anchorage-independent growth (6). In this model system, Y1440 was identified as the major binding site, with minor contributions from Y1257 and Y1494 (6). We have now demonstrated specific roles for Y1440 and Y1494 in the adhesion-dependent recruitment of SHP2 to the $\alpha 6 \beta 4$ receptor and have also identified a unique role for Y1494 in the activation of SHP2 catalytic activity, through its selective binding to the N-SH2 domain of SHP2. When Y1494 is not phosphorylated, SHP2 phosphatase activity is not increased by $\alpha 6 \beta 4$ ligation, likely because the N-SH2 domain remains bound to the catalytic cleft and blocks substrate access (Fig. 8). Binding of the N-SH2 domain to pY1494 is required to stimulate signaling downstream of SHP2 in response to $\alpha 6 \beta 4$ engagement.

We have identified a novel mechanism for the SHP2-dependent activation of Fyn by $\alpha 6 \beta 4$ that requires the recruitment of Fyn to pY580 in the SHP2 C-terminal tail. SFK activation is regulated through intramolecular interactions that are controlled by phosphorylation at inhibitory and stimulatory sites (36). Phosphorylation of the C-terminal tail (Y528 in human Fyn) inhibits activation by promoting an interaction of the N-terminal SH2 domain with this site. Dephosphorylation of the inhibitory tyrosine can disrupt this interaction and promote autophosphorylation in the activation loop (Y417 in human Fyn) to enhance catalytic activity. A number of studies have investigated the role of SHP2 in SFK activation, and both direct and indirect mechanisms have been reported. In response to growth factor stimulation, phosphorylation of the inhibitory Y527 in the C terminus of c-Src diminishes through a Shp2-dependent decrease in the recruitment of Csk kinase to the membrane domains where Src is localized. The transmembrane glycoprotein PAG and the focal adhesion protein paxillin, which are substrates of SHP2, have been implicated in this recruitment of Csk (34, 49). SHP2 has also been reported to activate Src independently of its catalytic function by binding directly to the SH3 domain of Src and disrupting an intramolecular interaction that interferes with the catalytic domain (45). We did not observe any changes in dephosphorylation of the inhibitory C-terminal tyrosine of Fyn in response to $\alpha 6 \beta 4$ engagement (data not shown). However, recruitment of Fyn to

Y580 in the C terminus of SHP2 via its SH2 domain would disrupt the intramolecular inhibition to allow autophosphorylation of the activation loop, even in the presence of persistent phosphorylation of the Fyn C terminus (14). Although our data support the idea that the catalytic activity of SHP2 contributes to Fyn activation in response to $\alpha 6 \beta 4$ engagement, the reduced ability of the C-terminal Y542F/Y580F double-mutant protein that retains full phosphatase activity to stimulate Fyn activation demonstrates that the physical interaction of SHP2 and Fyn is also essential for optimal activation of this pathway in response to $\alpha 6 \beta 4$ engagement.

Our elucidation of SHP2 function downstream of $\alpha 6 \beta 4$ adds to the mechanistic understanding of how this integrin receptor promotes carcinoma invasion. In previous studies, the ability of the $\alpha 6 \beta 4$ integrin to activate Fyn was shown to be dependent upon palmitoylation of the membrane-proximal region of the $\beta 4$ subunit (13, 48). However, the direct link between Fyn and the $\alpha 6 \beta 4$ receptor remained an open question. Fyn is also palmitoylated, which provides a mechanism to localize Fyn in the plasma membrane in proximity to the $\alpha 6 \beta 4$ integrin, where it can mediate signals at sites of adhesive contacts to promote motility and invasion (35). The potential importance of this localization is underscored by the fact that Src is not palmitoylated and therefore does not localize to the membrane domains containing $\alpha 6 \beta 4$, and it is not activated by this integrin receptor (35). Our current data now demonstrate that localization to membrane domains alone is not sufficient for Fyn activation because the recruitment of Fyn to pY580-SHP2 is required to activate Fyn in response to $\alpha 6 \beta 4$ engagement (Fig. 8). Our data also support a positive Fyn feedback loop that mediates the phosphorylation of Y542 in the C terminus of SHP2 to increase SHP2 catalytic activity. The fact that mutation of Y542 did not significantly reduce Fyn activation by $\alpha 6 \beta 4$ but did diminish invasion provides evidence that additional SHP2 substrates are likely to cooperate with Fyn to enhance $\alpha 6 \beta 4$ -dependent invasion. Interestingly, Fyn and SHP2 inversely regulate the activity of some signaling molecules that contribute to tumor invasion. For example, Fyn phosphorylates and activates p190RhoGAP to inactivate the small GTP-binding protein RhoA, whereas SHP2 dephosphorylates p190RhoGAP, thereby maintaining RhoA in an active GTP-bound state to stimulate downstream effectors (16, 38, 46). RhoA can directly influence cell motility and invasion through its regulation of the actin cytoskeleton, and the ability of Rho GTPases to cycle between active and inactive states is essential for this function (30). Engagement of the $\alpha 6 \beta 4$ integrin activates RhoA to promote lamella formation and migration (27). The formation of an active SHP2/Fyn complex would allow for the dynamic regulation of RhoA downstream of $\alpha 6 \beta 4$. Additional common downstream targets of Fyn and SHP2 that are likely to be important for promoting invasion include the focal adhesion components p130Cas and paxillin (24).

The enhanced phosphorylation of Y580-SHP2 and Y418-SFK in human orthotopic breast tumors that express WT- $\beta 4$ and the dependence of this SFK activation on Y1494 support the involvement of an $\alpha 6 \beta 4$ /SHP2/Fyn signaling pathway in carcinoma invasion and tumor progression. Breast tumors that express $\alpha 6 \beta 4$ have increased angiogenesis and enhanced metastasis compared with tumors that either lack expression of

this integrin receptor or express a mutant Y1494F- β 4 subunit (1, 10, 43). SFKs are expressed and activated in many tumor types, and numerous studies have demonstrated that SFK activity is associated with poor patient outcomes (15). In breast cancer, a Src-responsive gene signature that reflects active Src signaling has recently been found to be tightly associated with latent bone metastasis, and Src promotes growth and survival in the bone microenvironment (50). In contrast with SFKs, there is relatively little information regarding the connection of SHP2 with solid tumor progression, although many of the upstream growth factor and integrin receptors that signal through SHP2 have been implicated in cancer (8). Activating mutations in SHP2 have been identified in 35% of juvenile myelomonocytic leukemias (JMML), but the incidence of mutations in solid tumors is infrequent (5, 42). The majority of the JMML mutations disrupt the N-SH2 inhibitory interaction with the catalytic domain to increase basal phosphatase activity (9). Interestingly, JMML patients lacking SHP2 mutations have either deletion of the NF-1 gene or activating Ras mutations, suggesting an important role for SHP2 in the activation of Ras signaling in these tumors (5). SHP2 regulates Ras signaling through the negative regulation of RasGAP recruitment to signaling complexes, thereby leading to sustained Ras activation and downstream MAPK signaling (2, 25). Activation of SHP2 in response to engagement of α 6 β 4 through Y1494 could mimic activating SHP2 mutations to stimulate Ras signaling and promote tumor progression (20, 31). In support of this possibility, mutation of Y1494 diminishes α 6 β 4-dependent activation of MAPK signaling, which prevents anchorage-independent growth *in vitro* and tumor growth *in vivo* (10). Therefore, in addition to regulating SFK activation to promote invasion, the α 6 β 4-dependent recruitment and activation of SHP2 are likely to contribute to multiple signaling pathways that promote tumor progression.

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