The regulation of Rho and Src signaling by integrins
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Integrin αvβ3 Controls Activity and Oncogenic Potential of Primed c-Src

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Increased activity of the proto-oncogene c-Src and elevated levels of integrin αvβ3 are found in melanomas and multiple carcinomas. Regulation of c-Src involves “priming” through disruption of intramolecular interactions followed by “activation” through phosphorylation in the kinase domain. Interactions with overexpressed receptor tyrosine kinases or mutations in the SRC gene can induce priming of c-Src in cancer. Here, we show that αvβ3 promotes activation of primed c-Src, causing enhanced phosphorylation of established Src substrates, survival, proliferation, and tumor growth. The β3 cytoplasmic tail is required and sufficient for integrin-mediated stimulation of all these events through a mechanism that is independent of β3 tyrosine phosphorylation. Instead, experiments using Src variants containing the v-Src Src homology 3 (SH3) domain and using mutant β3 subunits indicate that a functional interaction of the β3 cytoplasmic tail with the c-Src SH3 domain is required. These findings delineate a novel integrin-controlled oncogenic signaling cascade and suggest that the interaction of αvβ3 with c-Src may represent a novel target for therapeutic intervention.

INTRODUCTION

Interactions of tumor cells with their microenvironment are important for cancer development and progression (Bissell and Radisky, 2001). Tumor cells connect with the extracellular matrix (ECM) through various members of the integrin family of adhesion receptors and upon malignant transformation cells often undergo specific changes in the expression levels of integrins. High levels of integrin αvβ3 correlate with growth and/or progression of melanoma (Albelda et al., 1990; Hsu et al., 1998), neuroblastoma (Gladson et al., 1996) and multiple different carcinomas (Chattopadhyay and Chatterjee, 2001; Liapis et al., 1997; Pignatelli et al., 1992; Sengupta et al., 2001; Vonlaufen et al., 2001). Moreover, individuals homozygous for the β3L33P polymorphism that enhances the affinity of β3 integrins, have an increased risk to develop breast cancer, ovarian cancer, and melanoma (Bojesen et al., 2003). Despite the fact that αvβ3 in the tumor vasculature has been identified as a valuable drug target, endothelial αvβ3 is dispensable for tumorigenesis (Reynolds et al., 2002; Taverna et al., 2005). It remains unclear if and how increased levels of αvβ3 on tumor cells contribute to cancer development.

Following ligand binding, integrins cluster and organize into multi-protein complexes termed cell-matrix adhesions that connect to the actin cytoskeleton through a variety of cytoskeletal linker proteins. Cell-matrix adhesions also contain various signaling intermediates, including non-receptor tyrosine kinases such as focal adhesion kinase (FAK) and c-Src (Geiger et al., 2001). Integrin-mediated adhesion stimulates FAK and c-Src activities and, in turn, c-Src modulates the stability of cell-matrix adhesions through phosphorylation of several components, including integrin
cytoplasmic tails (Arias-Salgado et al., 2003; Haimovich et al., 1991; Sakai et al., 2001). In addition, the FAK/c-Src complex is involved in the transmission of information from the ECM into the cell to regulate cellular signaling cascades in control of apoptosis and proliferation (Danen and Yamada, 2001).

In unstimulated cells, c-Src is folded into a closed, autoinhibitory conformation. Its activation requires dephosphorylation of the C-terminal Tyr530 residue (amino acid numbering used in this paper is for human c-Src) to disrupt intramolecular binding of this residue to the SH2 domain. A disruption of the interaction between the SH3 domain and prolines in the linker region further contributes to the formation of an unfolded or “primed” conformation. Finally, for full enzymatic activity, primed c-Src must be phosphorylated in its kinase domain at residue Tyr419 by transphosphorylation (Martin, 2001; Thomas and Brugge, 1997). The oncogenic product of Rous sarcoma virus, v-Src, is constitutively activated through amino acid substitutions in the SH3 domain and the kinase domain as well as a deletion of the regulatory C-terminal tyrosine (Jove and Hanafusa, 1987; Martin, 2001). While Rous sarcoma virus is avian specific, c-Src plays a critical role in cancer development (Guy et al., 1994). Indeed, levels of c-Src activity are frequently increased in human melanoma and carcinomas of the breast, colon, and other epithelia (Irby and Yeatman, 2000; Ishizawar and Parsons, 2004; Liapis et al., 1997; Niu et al., 2002; Pignatelli et al., 1992; Sengupta et al., 2001; Vonlaufen et al., 2001). We hypothesized that a functional interaction of αvβ3 with c-Src may contribute to cancer development. In this report we show that the activity and oncogenic potential of primed c-Src is in fact subject to a remarkably tight regulation by integrin αvβ3. Our findings identify the β3 cytoplasmic domain as a critical regulator of c-Src-mediated oncogenic signaling.

MATERIALS AND METHODS

Cell lines and plasmids

The HBL100 cell line was obtained from ATCC. The β1 integrin-deficient cell lines GD25 and GE11 were provided by Dr. Reinhard Fässler (Max Planck Institute, Martinsried, Germany) and have been described previously (Danen et al., 2002; Albrecht et al., 2004). All cell lines were cultured in DMEM supplemented with 10% fetal calf serum, penicillin and streptomycin. A Myc tag was added at the 3′ end of the cDNA encoding c-SrcV530E (the plasmid encoding mouse-chicken c-Src in which the C-terminal regulatory Tyr was replaced by Phe was purchased from Upstate Biotechnology), an HA tag was added at the 3′ end of the cDNA encoding mouse c-Src (Upstate Biotechnology), and the tagged constructs were cloned into the LZRS retroviral vector. Retroviral expression plasmids encoding integrin β1 or β3 subunits, β1β3 and β3β1 chimeras, and those encoding the extracellular and transmembrane region of the non-signaling IL2 receptor (IL2R) α subunit alone or fused to the integrin β1 cytoplasmic domain were described before (Danen et al., 2002; Albrecht et al., 2004). To generate the LZRS-IL2Rβ3 plasmid, the β1 cytoplasmic domain in IL2Rβ1 was replaced with the β3 cytoplasmic domain. The retroviral expression plasmid encoding ts72v-Src (Mossoglu and Gelman, 2003) was provided by Dr. Irwin H. Gelman (Roswell Park Cancer Institute, Buffalo, NY, USA). The
LZRS retroviral construct expressing chimeric vSrc/SrcYF was generated by substituting the first 131 amino acids of SrcYF (containing an N-terminal Myc tag) with the same region from ts72v-Src. The cDNA encoding human EGFR was provided by Dr. Frank Furnari (Ludwig Institute for Cancer Research, La Jolla, CA, USA) and cloned into the pMSCV retroviral expression plasmid by Ms. Sophia Bruggeman (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The retroviral H-RasG12V expression plasmid (RasGV) was provided by Dr. John Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The β3Y747A, β3Y759A and β3Y759 mutants were provided by Dr. Jari Ylänne (University of Oulu, Finland) and subcloned into the LZRS retroviral vector (Ylänne et al., 1995). All cDNAs were transfected into amphotrophic or ecotrophic packaging cells to generate virus-containing culture supernatants that were used for retroviral transduction of HBL100, GD25, and GE11 cells.

Figure 1. Integrin αvβ3 supports oncogenic transformation by SrcYF. (A, C) Western blot analysis of SrcYF (Myc-tag antibody), total Src (c-Src antibody), and β-actin in lysates of GD25 (A) or GE11 cells (C) expressing the indicated constructs. Note that lysates were separated on a 10% polyacrylamide gel in (C), allowing the visualization of c-Src and the Myc-tagged SrcYF as separate bands (middle panel), whereas 4-20% gels were used in all other cases. (B, D) Colony formation and tumorigenicity of GD25 (B) or GE11 cells (D) expressing SrcYF and αvβ3 integrins as indicated. Phase-contrast images of soft agar assays were taken 14 days after plating; quantification shows average number of colonies larger than 5 cells per image ± SEM (standard error of the mean) of at least two independent experiments. The small clusters of GDSrcYF/β1 cells (smaller than 5 cells) did not grow out with time. Tumor growth curves show average tumor volume ± SEM of 1×10^6 injected cells; n=4 for GD25 lines, n=11 for GE11 lines obtained from two independent experiments.
Subsequently, Src<sup>YF</sup>, c-Src, ts72v-Src, vSrc/Src<sup>YF</sup>, or Ras<sup>YF</sup> expressing clones were transduced with retroviral constructs encoding wild-type, mutant, and chimeric integrin subunits or EGFR. Positive cells were bulk sorted at least twice by FACS for human integrin, IL2Rα, or EGFR surface expression.

**Antibodies and other materials**

Anti-human β1 monoclonal antibodies were TS2/16, clone 18 (BD Transduction Laboratories), and K20 (Biomeda). Anti-human β3 monoclonal antibodies were C17 (provided by Dr. Ellen van der Schoot, Sanquin, Amsterdam, The Netherlands), 23C6 (provided by Dr. Michael Horton, University of Manchester, UK), and SSA6 (provided by Dr. Sanford Shattil, University of California San Diego, CA, USA). Other monoclonal antibodies were anti-c-Src (B-12, Santa Cruz), anti-FAK<sup>pY397</sup> (Biosource), anti-FAK (C-20, Santa Cruz), anti-myc (A-14, Santa Cruz), anti-HA (GeneTex Inc.), anti-FAK (C-925, Santa Cruz), anti-EGFR (3E2, Cell Signalling Technology) and anti-BrdU (Bu20a, DAKO). The following rabbit polyclonal antibodies were used: anti-Src<sup>pY416</sup> (Bu20a, DAKO), anti-Stat3<sup>pY705</sup> (K-15, Santa Cruz), anti-Stat3 (K-15, Santa Cruz), anti-c-Src<sup>pY530</sup> (Biosource), anti-c-Src (SRC 2, Santa Cruz), anti-α-tubulin (B-5-1-2, Sigma), anti-β-actin (AC-15, Sigma), anti-EGFR (Ab-1 clone 528, Calbiochem), anti-Stat3<sup>pY705</sup> (3E2, Cell Signalling Technology) and anti-BrdU (Bu20a, DAKO). The following rabbit polyclonal antibodies were used: anti-Src<sup>pY416</sup> (Biosource), anti-c-Src (SRC 2, Santa Cruz), anti-α-actin (AC-15, Sigma), anti-EGFR (Ab-1 clone 528, Calbiochem), anti-Stat3<sup>pY705</sup> (3E2, Cell Signalling Technology) and anti-BrdU (Bu20a, DAKO). The following rabbit polyclonal antibodies were used: anti-Src<sup>pY416</sup> (Biosource), anti-c-Src (SRC 2, Santa Cruz), anti-α-tubulin (B-5-1-2, Sigma), anti-β-actin (AC-15, Sigma), anti-EGFR (Ab-1 clone 528, Calbiochem), anti-Stat3<sup>pY705</sup> (3E2, Cell Signalling Technology) and anti-BrdU (Bu20a, DAKO).

Flow cytometry, immunofluorescence, and Western blot analysis

These experiments were performed as described previously (Danen et al., 2002).

**Immunoprecipitations**

For immunoprecipitations cells were lysed for 15 minutes at 4°C in lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH7.4), 150 mM NaCl, 1 mM sodium vanadate, 0.5 mM sodium fluoride and protease inhibitor cocktail (Sigma-Aldrich)). Lysates were clarified by centrifugation at 14,000 rpm for 15 minutes at 4°C. Src<sup>YF</sup> protein was isolated from the clarified lysates by immunoprecipitation with 5 μg anti-myc antibody for two hours at 4°C, and immune complexes were collected with protein A-Sepharose. Kinase activity of isolated Src<sup>YF</sup> protein was determined by use of a Src kinase assay kit (Upstate Cell Signaling Solutions).

**Soft-agar and tumorigenicity assays**

For soft agar assays, six-well plates were first coated with 0.6% low melting point (LMP) agarose (Roche). Subsequently, 100,000 cells were suspended in culture medium containing 0.35% LMP agarose and seeded on top of the 0.6% LMP agarose layer. For tumorigenicity assays, cells were harvested, washed and resuspended in 0.2 ml sterile PBS per injection. Female 6-weeks-old athymic Balb/c mice were then subcutaneously injected into the left and right flanks. After cell inoculation, tumor volumes were measured using calipers at the indicated times. All animal experiments were approved by the animal welfare committee of the Netherlands Cancer Institute.

**TUNEL staining and BrdU incorporation assays**

75,000 cells were plated in culture medium on fibronectin coated coverslips and after 4 hours the cells were either kept in culture medium or switched to serum-free medium for 24 or 48 hours. The cells were labeled with 15 μM BrdU (Sigma) for 4 hours before fixation in 2% paraformaldehyde. For BrdU staining, cells were permeabilized in 0.5% Triton X-100, DNA was denatured with 2M HCl and neutralized with 0.1M sodium borate, and coverslips were labeled with anti-BrdU antibody followed by FITC-conjugated secondary antibody. For TUNEL staining, cells were permeabilized in 0.1% Triton X-100 with 0.1% sodium citrate in PBS and stained with an in situ cell death detection kit (Roche). For both procedures, nuclei were visualized with TOPRO-3 (Molecular Probes) and preparations were mounted in MOWIOL 4-88 solution supplemented with DABCO (Calbiochem).

**RESULTS**

Integrin αvβ3 supports primed c-Src-mediated tumor growth

A c-Src mutant in which a primed conformation is induced by the substitution of Tyr<sup>530</sup> by Phe (Src<sup>-</sup>) was introduced alone or
αvβ3 controls c-Src-mediated tumor growth

Figure 2. Specific cooperation of αvβ3 and primed c-Src stimulates tumor growth in a cell-autonomous fashion. (A) Growth curves showing average tumor volume ± SEM of GESrc<sup>YF</sup>β3 cells lacking (filled circles) or expressing β1 (open squares); n=8 obtained from two independent experiments where 1×10<sup>6</sup> cells were injected. (B) Quantification of integrin expression on cells isolated from 4 tumors of a 1:1 mix of 5×10<sup>5</sup> GESrc<sup>YF</sup>β1 and GESrc<sup>YF</sup>β3 cells ± SEM. Expression of human β1 (open bars) and human β3 integrins (filled bars) was determined by FACS before injection (“input”) or after one month of tumor growth (“tumors”). The fact that the percentage of GESrc<sup>YF</sup>β1 and GESrc<sup>YF</sup>β3 cells does not add up to 100% can be explained by the presence of stromal cells (lacking human β1 and β3 integrins). (C) Growth curves showing average tumor volume ± SEM of GERas<sup>GF</sup>β1 (open squares) and GERas<sup>GF</sup>β3 cells (filled circles); n=7 obtained from two independent experiments where 1×10<sup>6</sup> cells were injected. (D) Average tumor volume ± SEM at 33 days post injection of GEβ1 (open bars) and GEβ3 cells (filled bars) containing the indicated expression constructs. n=12 obtained from two independent experiments where 1×10<sup>6</sup> cells were injected. Asterisk indicates significant difference between the mean values (Student’s T test, P<0.01).

in combination with the β3 integrin subunit in HBL100, GD25, and GE11 cells. While Src<sup>YF</sup>-transformed HBL100 cells were tumorigenic, tumors grew much faster when the surface expression levels of αvβ3 were increased (Supplementary Figure 1). The cooperation between αvβ3 and Src<sup>YF</sup> was even more striking in GD25 and GE11: while cells expressing β1 integrins (GDSrc<sup>YF</sup>β1 and GESrc<sup>YF</sup>β1) were virtually unable to grow in soft-agar or form tumors in mice, cells lacking β1 integrins but expressing high levels of αvβ3 (GDSrc<sup>YF</sup>β3 and GESrc<sup>YF</sup>β3) were highly tumorigenic (Figure 1; Supplementary Figure 2A and see [Danen et al., 2002]). Expression of β1 integrins in GESrc<sup>YF</sup>β3 cells did not affect tumor growth indicating that αvβ3 supports Src<sup>YF</sup>-mediated tumor formation, irrespective of the expression of β1 integrins (Figure 2A). Moreover, when a 1:1 mixture of GESrc<sup>YF</sup>β1 and GESrc<sup>YF</sup>β3 cells was injected subcutaneously, GESrc<sup>YF</sup>β3 cells (recognized by an antibody directed against human β3) were readily
Figure 3. The β3 cytoplasmic tail supports Src\textsuperscript{YF} activation and Src\textsuperscript{YF}-mediated tumor growth. (A) Western blot analysis of p-Stat3(Y\textsuperscript{705}), total Stat3 (α and β isoforms), and tubulin loading control in lysates of GE\textsuperscript{β1} and GE\textsuperscript{β3} cells expressing or lacking Src\textsuperscript{YF}. Dotted lines in A and B separate different regions from a single film placed together. (B) Western blot analysis of p-Src(Y\textsuperscript{419}) and total Src in lysates of GE\textsuperscript{β1} and GE\textsuperscript{β3} cells expressing or lacking Src\textsuperscript{YF}. (C) Western blot analysis of p-Src(Y\textsuperscript{419}), total Src, p-FAK(Y\textsuperscript{925}), total FAK, p-Stat3(Y\textsuperscript{705}), total Stat3 and loading control in lysates of GE\textsuperscript{11} cells expressing the indicated constructs and grown in the absence of serum. Quantification shows mean ± SEM of relative p-Src(Y\textsuperscript{419}), p-FAK(Y\textsuperscript{925}) and p-Stat3(Y\textsuperscript{705}) levels compared to GESrc\textsuperscript{YF}/GE\textsuperscript{β1} in serum-starved cultures of GE\textsuperscript{11} cells expressing the indicated constructs obtained from at least two independent experiments. (D) Activity of Src\textsuperscript{YF} immunoprecipitated from GE\textsuperscript{11} (--), GESrc\textsuperscript{YF}/GE\textsuperscript{β1} or GESrc\textsuperscript{YF}/GE\textsuperscript{β3} cells in an in vitro Src kinase assay. (E) Growth curves showing average tumor volume ± SEM of GESrc\textsuperscript{YF}/β1*3 (filled circles) or GESrc\textsuperscript{YF}/β3*1 cells (open squares); n=8 obtained from two independent experiments where 1×10\textsuperscript{6} cells were injected.

detectable in the resulting tumors whereas GESrc\textsuperscript{YF}/β1 cells (recognized by an antibody directed against human β1) were virtually absent, indicating that αvβ3 supports Src-mediated tumor formation in a cell-autonomous fashion (Figure 2B).

In contrast to Src\textsuperscript{YFF}, Ras\textsuperscript{GTV}-mediated tumor growth was not affected by the expression of αvβ3, indicating that this integrin is specifically required for Src-mediated tumorigenesis (Figure 2C). In several human cancers, overexpressed and/or activated EGF receptors (EGFRs) can stimulate priming of c-Src through (in)direct interactions with its SH2 domain (Irby and Yeatman, 2000; Thomas and Brugge, 1997). We analyzed if αvβ3 can also support c-Src-mediated tumor growth under such conditions. Moderate overexpression of c-Src was not sufficient by itself to induce tumor
formation even in the presence of αvβ3 (Figure 2D; Supplementary Figure 2B). However, even though tumors grew slow compared to those induced by SrcYF, αvβ3 significantly increased tumor growth when c-Src and EGFR were co-expressed (Figure 2D; Supplementary Figure 2C).

Together, these findings indicate that αvβ3 specifically cooperates with primed c-Src in a cell-autonomous fashion to stimulate the formation of tumors by fibroblasts and epithelial cells.

αvβ3 supports SrcYF-mediated survival, proliferation, and tumor formation upstream of FAK and Stat3 by enhancing SrcYF activation

The activation of the transcription factor Stat3 by phosphorylation at Tyr 705 is strongly enhanced in cells transformed by v-Src (Bromberg et al., 1998; Turkson et al., 1998). SrcYF only moderately increased Stat3 activity in the presence of β1 integrins whereas phosphorylation was clearly enhanced in the presence of αvβ3 (Figure 3A). This suggested that αvβ3 may enhance SrcYF activity, which, like wild-type c-Src, requires transphosphorylation of Tyr 419 in its catalytic domain to acquire full enzymatic activity. Indeed, while expression of SrcYF led to increased levels of Src[pY419] in the presence of either β1 or β3 integrins, phosphorylation was much stronger in GESrcYFβ3 than in GESrcYFβ1 cells (Figure 3B). Stimulation of the levels of Src[pY419] and of two known Src substrates, FAK[pY925] and Stat3[pY705] in the presence of αvβ3 was also observed when cells were cultured under conditions that may mimic the tumor environment (serum-free or non-adherent) (Figure 3C and data not shown). On the other hand, equal levels of Src activity were detected in in vitro Src kinase assays on SrcYF immunoprecipitates from GESrcYFβ1 and GESrcYFβ3 lysates (Figure 3D). These data raise the possibility that in vivo activation of SrcYF is regulated by αvβ3, possibly by enhanced clustering and subsequent transphosphorylation in the kinase domain. Concentration of SrcYF on the beads in the in vitro Src kinase assays might result in activation of SrcYF in a similar way.

Figure 4. The β3 cytoplasmic tail supports SrcYF signaling to survival and proliferation. (A) TUNEL assays on GE11 cells expressing the indicated constructs under standard culture conditions and after 24 and 48 hours of serum starvation. Graph shows mean percentage of TUNEL positive cells ± SEM of two independent experiments. (B) BrdU incorporation under conditions described for (A). Graph shows mean percentage of BrdU positive cells ± SEM of three independent experiments. Asterisks indicate significant difference between the mean values (Student’s T test, P<0.05).
To investigate the role of the β3 cytoplasmic tail in SrcYF activation and oncogenic potential, we expressed a chimeric integrin subunit consisting of the β3 extracellular and transmembrane regions fused to the β1 cytoplasmic region (β3exβ1in) in GESrcYF cells (Supplementary Figure 2A, left panel). Unlike wild type β3, β3exβ1in did not enhance the levels of Src[pY419], Stat3[pY705] and FAK[pY925] (Figure 3C). On the other hand, an inverse chimeric β1inβ3ex integrin subunit, unlike wild type β1, strongly increased the degree of phosphorylation of Src, Stat3 and FAK (Figure 3C; Supplementary Figure 2A, right panel). Moreover, surface expression of β1inβ3ex supported SrcYF-mediated tumor formation whereas expression of β3exβ1in did not (Figure 3E).

We next investigated if increased levels of αvβ3 promote survival and proliferation of SrcYF transformed cells. After 24 and 48 hours of serum deprivation there were significantly fewer GESrcYF β3 than GESrcYF β1 cells (Figure 4A, Supplementary Figure 3A, P<0.05 in Student’s T test). In agreement with the role of the β3 cytoplasmic tail in tumor formation induced by SrcYF, the sensitivity to serum starvation was suppressed in the presence of the β1exβ3 in but not the β3exβ1 in chimeric integrin subunit (P<0.05). Furthermore, following serum deprivation the expression of β3 or β1exβ3 in correlated with high proliferation rates whereas a large proportion of the cells expressing β1 or β3exβ1 in underwent cell cycle arrest (P<0.05) (Figure 4B, Supplementary Figure 3B).

From these findings we conclude that: i) the primed SrcYF mutant is in fact subject to tight regulation in vivo, ii) as part of a functional integrin the β3 cytoplasmic tail is required and sufficient to support the activity of SrcYF under conditions that mimic the tumor environment, and iii) the ability of the β3 cytoplasmic tail to support SrcYF activation is correlated with its ability to support SrcYF-mediated survival, proliferation, and tumor formation.

Functional, spatial, and molecular association of the β3 cytoplasmic tail with SrcYF

Having demonstrated that the β3 cytoplasmic tail controls the activity and oncogenic potential of SrcYF, we asked two questions: is SrcYF-mediated phosphorylation of the β3 tail involved and can the β3 tail on its own support oncogenic signaling by primed Src? Both β3Y747A and β3Y759A mutants stimulated tumor growth of GESrcYF cells to the same extent as wild type β3 indicating that recruitment of signaling and adaptor proteins such as Shc and Grb2 to phosphorylated tyrosine residues in the β3 cytoplasmic tail is not required (Figure 5A; Supplementary Figure 4A). On the other hand an IL2R fusion construct containing the β3 cytoplasmic tail did not enhance tumor growth of GESrcYF cells compared to IL2R- or IL2Rβ1, indicating that the cooperation between the β3 cytoplasmic tail and SrcYF requires the context of a functional integrin (Figure 5B; Supplementary Figure 4B).

We next investigated if SrcYF formed a complex with the β3 subunit but did not detect an interaction using co-immunoprecipitations while an interaction of SrcYF with endogenous FAK was readily detectable (Figure 5C; Supplementary Figure 4C; data not shown). To investigate if a possible weak interaction may occur we analyzed the subcellular localization of SrcYF and β3 integrins. Irrespective of the type of integrins expressed, SrcYF induced the formation of podosomes, adhesive structures that are characteristic for Src transformed cells. Notably, this indicates that the activity of SrcYF in cells lacking high amounts of αvβ3 is sufficient to cause morphological, but not oncogenic transformation. β1 and β3 integrins were partially co-localized with SrcYF in podosomes (Figure 5D, left and middle panels) making it possible that the β3 cytoplasmic tail locally interacts with SrcYF and enhances SrcYF-mediated oncogenic signaling. In line with this idea, IL2Rβ3 did not co-localize with SrcYF in podosomes, which may explain its inability to support
\( \alpha v \beta 3 \) controls c-Src-mediated tumor growth

**Figure 5. Functional and spatial association of the \( \beta 3 \) cytoplasmic tail and Src\(^{YF} \).** (A) Growth curves showing average tumor volume ± SEM of GESrc\(^{YF} \) cells expressing integrin \( \beta 3 \) (filled circles), \( \beta 3^{Y747A} \) (open squares), or \( \beta 3^{Y759A} \) (filled squares); \( n=12 \) obtained from two independent experiments where \( 1 \times 10^6 \) cells were injected. (B) Growth curves showing average tumor volume ± SEM of GESrc\(^{YF} \) cells expressing integrin \( \beta 3 \) (filled circles), IL2R\(^{-} \) (filled triangles), IL2R\(^{1} \) (open circles) or IL2R\(^{3} \) (open squares); \( n=5 \) obtained from at least two independent experiments where \( 1 \times 10^6 \) cells were injected. (C) Western blot analysis of the indicated proteins in immunoprecipitations of \( \beta 3 \) (upper panel) or \( \beta 1 \) integrin (lower panel), or in total lysates of GESrc\(^{YF} \) and GESrc\(^{YF} \) cells (WCL; whole cell lysate). (D) Localization of Src\(^{YF} \) (anti-Myc antibody; green), integrin \( \beta 1 \), \( \beta 3 \) or IL2R\(^{3} \) (K20, 23C6, anti-IL2R\( \alpha \) antibodies respectively, Texas red) in GE11 cells expressing the indicated constructs. Arrows indicate co-localization. Bars, 5 \( \mu m \).

Src\(^{YF} \)-mediated oncogenic transformation (Figure 5D, right panel).

The last four amino acids of the integrin \( \beta 3 \) tail have been reported to mediate binding of \( \alpha I I b \beta 3 \) to the SH3 domain of c-Src (Arias-Salgado et al., 2003). A similar interaction may explain \( \alpha v \beta 3 \)-mediated control of the oncogenic potential of primed...
c-Src. Experiments using v-Src, which contains multiple mutations in its SH3 domain (Martin, 2001) showed efficient phosphorylation on Tyr419 and colony outgrowth in soft-agar irrespective of αvβ3 expression levels (Figure 6A,B). However, v-Src also contains activating mutations in its kinase domain (Martin, 2001) that may make the interaction with the β3 cytoplasmic domain redundant. Therefore, we generated a v-Src/SrcYF chimera in which only the N-terminal region including the SH3 domain was derived from v-Src. This construct failed to induce oncogenic transformation even in the presence of high levels of αvβ3 (Figure 6C; Supplementary Figure 4D). Moreover, expression of a β3759 mutant that lacks the four most C-terminal amino-acids required for binding the c-Src SH3 domain (Arias-Salgado et al., 2003) failed to support SrcYF-mediated tumor formation (Figure 6D).

Together, these data support the idea that oncogenic activity of primed Src variants containing a wild type kinase domain depends on SH3-mediated interactions with the β3 cytoplasmic domain.

**Figure 6.** Cooperation between SrcYF and αvβ3 involves integrin interaction with the Src SH3 domain. (A) Western blot analysis of p-Src(Y419), total Src, and actin loading control in lysates of GEβ1 and GEβ3 cells expressing ts72v-Src at non-permissive (NPT) and permissive temperature (PT). (B) Colony formation of GEts72v-Srcβ1 and GEts72v-Srcβ3 cells. Phase-contrast images of soft agar assays were taken 10 days after plating at PT; quantification shows average number of colonies larger than 5 cells per image ± SEM of two independent experiments. (C) Growth curves showing average tumor volume ± SEM of GEβ1 cells expressing the indicated constructs; n=10 obtained from two independent experiments where 1×10⁶ cells were injected. (D) Growth curves showing average tumor volume ± SEM of GESrcβ3 (filled circles) or GESrcβ3759 cells (open squares); n=5 obtained from two independent experiments where 1×10⁶ cells were injected.
DISCUSSION

In human melanomas and carcinomas of the breast, colon, pancreas, and other organs, the activity of c-Src is frequently increased compared to that in surrounding tissue (Irby and Yeatman, 2000; Ishizawar and Parsons, 2004; Niu et al., 2002). It is not fully understood how high levels of c-Src activity contribute to human cancer. The fact that activating mutations in the SRC gene appear to be rare argues against a role in tumor initiation. Increased c-Src activity may contribute to invasion and metastasis by promoting tumor cell scattering, migration, proteolytic activity, and anoikis-resistance (Frame, 2004; Summy and Gallick, 2003). For colon cancer, increased c-Src activity may also contribute to tumor growth (Irby et al., 1997), perhaps by stimulating VEGF-mediated angiogenesis (Ellis et al., 1998).

Our findings clearly demonstrate that elevated c-Src activity promotes tumorigenicity of immortalized cells where the p53 and Rb tumor suppressor pathways are suppressed, as is almost invariably the case in human cancer. We find that elevated c-Src activity promotes tumor growth in a cell-autonomous fashion by stimulating survival and proliferation. This may be especially important during early stages of cancer development. The contribution of elevated levels of c-Src activity to tumor growth may decrease as tumors progress and acquire additional mutations, e.g. those activating Ras.

The molecular mechanism responsible for the increased activity of c-Src in human cancers is incompletely understood. Overexpression of RTKs has been proposed to induce a primed conformation of c-Src by disrupting the intramolecular binding of the SH2 domain to phosphorylated Tyr530 (Irby and Yeatman, 2000; Thomas and Brugge, 1997). In addition, mutations in the C-terminal region of the SRC gene that lead to a primed conformation of c-Src have been detected in a small subset of carcinomas of the colon and the endometrium (Irby et al., 1999; Sugimura et al., 2000). Whatever the mechanism of priming, our findings demonstrate that the oncogenic potential of primed c-Src can be strongly enhanced by integrin αvβ3. The notion that αvβ3 and c-Src may cooperate in human cancer is supported by a number of reports showing that an increase in the expression of αvβ3 is associated with growth and/or progression of various cancers in which c-Src activity is frequently enhanced (Albelda et al., 1990; Chattopadhyay and Chatterjee, 2001; Gladson et al., 1996; Hsu et al., 1998; Irby and Yeatman, 2000; Ishizawar and Parsons, 2004; Liapis et al., 1997; Niu et al., 2002; Pignetelli et al., 1992; Sengupta et al., 2001; Vonlaufen et al., 2001). The loss of α5β1 or other β1 integrins has also been associated with oncogenic transformation and tumor growth (Brakebusch et al., 1999; Giancotti and Ruoslahti, 1990). We observed that αvβ1-deficient SrcYF transformed cells were slightly more tumorigenic than their αvβ1-expressing derivatives (data not shown). However, expression of β1 in SrcYFβ3 cells did not reduce their tumorigenic capacity, indicating that αvβ3 supports oncogenic signaling by primed Src irrespective of the expression of β1 integrins. The expression of αvβ3 will be important for Src-mediated aspects of cancer development while αvβ3 may be dispensable for those aspects that are driven by oncogenic Ras (our findings) or other oncogenes such as c-Neu (Taverna et al., 2005).

The interaction between the β3 cytoplasmic tail and the c-Src SH3 domain has been demonstrated by others (Arias-Salgado et al., 2003) but we were unable to detect the interaction of the β3 tail and primed c-Src by co-immunoprecipitation (possibly due to the much lower levels of expression). Nevertheless, several lines of evidence support a model in which this interaction controls the oncogenic potential of primed c-Src: i) only those integrins and integrin chimeras that contain the β3 cytoplasmic tail promote oncogenic signaling by primed c-Src; ii) in contrast to full length β3 an IL2R-β3 fusion construct fails to co-
localize with primed Src in podosomes and fails to support tumor growth. iii) the YRGT motif in the β3 cytoplasmic domain that was reported to interact with the SH3 domain of c-Src (Arias-Salgado et al., 2003) is required for the functional interaction of αvβ3 with primed c-Src; iv) αvβ3 cannot stimulate primed c-Src variants containing the SH3 domain of v-Src despite the fact that both SH2- and SH3-mediated autoinhibition is prevented. The β3 subunit has a tendency to form homo-oligomers and clustering of αvβ3 in the plane of the membrane may co-cluster primed c-Src leading to enhanced activation through cross-phosphorylation in the kinase domain (Li et al., 2003; Shattil, 2005). Indeed, such intermolecular auto-phosphorylation is considered to be the major mechanism underlying c-Src activation (Cooper and MacAuley, 1988). In addition to clustering, αvβ3 may support conformational alterations in the Src protein or recruit additional proteins that contribute to oncogenic signaling. In this respect, our results using Tyr to Ala mutants argue against a role for the recruitment of signaling or adaptor proteins to the conserved NpxY/NxxY motifs in the β3 cytoplasmic tail.

In conclusion, a functional interaction with the β3 cytoplasmic tail augments the activity and oncogenic potential of primed c-Src. Phosphorylation of FAK and Stat3 are enhanced in the presence of αvβ3 but it remains to be investigated if these or other downstream pathways underlie the synergistic effect of primed c-Src and αvβ3 on survival, proliferation, and tumor growth. As overexpression of αvβ3 and elevated levels of c-Src activity occur in the same types of tumors, the interaction of these two proteins may be an important event in cancer development and/or progression. Interfering with their interaction might therefore be a valuable therapeutic approach in melanomas and carcinomas of the breast, colon, and several other tissues. Moreover, a combinatorial analysis of the levels of integrin αvβ3 and c-Src may be useful to predict cancer development and/or progression.

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SUPPLEMENTARY FIGURES

Supplementary Figure 1. (A) FACS analysis of integrin αvβ3 on HBL100Src[YF] and HBL100Src[YF]/β3 cells (cIg, control immunoglobulin; Ab, antibody). (B) Western blot analysis of Src[YF] (Myc-tag antibody) and total Src (c-Src antibody) in lysates of HBL100 cells containing the indicated expression constructs. (C) Growth curves showing tumor volume of $1 \times 10^6$ (upper panel) or $5 \times 10^5$ (lower panel) subcutaneously injected HBL100Src[YF] (open squares) and HBL100Src[YF]/β3 (filled circles) cells. Average tumor volume ± SEM of 4 injections is shown.
Supplementary Figure 2. (A) FACS analysis of human β3 (left) and β1 (right) integrins on GE11 cells expressing the indicated constructs. (B) Western blot analysis of exogenous c-Src (HA-tag antibody), Src<sup>YF</sup> (Myc-tag antibody), total Src (c-Src antibody), and β-actin loading control in lysates of GE11 cells expressing the indicated constructs. (C) FACS analysis of human EGFR on GE11 cells expressing the indicated constructs.

Supplementary Figure 3. Representative images of TUNEL assays (A) and BrdU incorporation (B) in GE11 cells expressing the indicated constructs under standard culture conditions and after 24 and 48 hours of serum starvation. TUNEL or BrdU positive cells are stained in green; nuclei are visualized with TOPRO-3 (red). Bars, 50 μm.
Supplementary Figure 4. (A) FACS analysis of human β3 integrins on GE11 cells expressing the indicated constructs. (B) FACS analysis of human IL2Rα on GE11 cells expressing the indicated constructs. (C) Western blot analysis of the indicated proteins in total lysates and Src\textsuperscript{YF} immunoprecipitations from GESrc\textsuperscript{YF}β1 and GESrc\textsuperscript{YF}β3 cells (asterisk indicates signal from previous incubation with anti-β3 antibody). (D) Western blot analysis of Src\textsuperscript{YF} and vSrc/Src\textsuperscript{YF} (Myc-tag antibody), total Src (c-Src antibody), and β-actin loading control in lysates of GE11 cells expressing the indicated constructs.