Activated Src increases adhesion, survival and α2-integrin expression in human breast cancer cells

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Focal adhesion kinase (FAK) is an intracellular kinase that localizes to focal adhesions. FAK is overexpressed in human tumours, and FAK regulates both cellular adhesion and anti-apoptotic survival signalling. Disruption of FAK function by overexpression of the FAK C-terminal domain [FAK-CD, analogous to the FRNK (FAK-related non-kinase) protein] leads to loss of adhesion and apoptosis in tumour cells. We have shown that overexpression of an activated form of the Src tyrosine kinase suppressed the loss of adhesion induced by dominant-negative; adenoviral FAK-CD and decreased the apoptotic response in BT474 and MCF-7 breast cancer cell lines. This adhesion-dependent apoptosis was increased by the Src-family kinase inhibitor PP2 {4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine}. We have also shown that expression of activated Src in breast cancer cells increased the expression of α2-integrin and that overexpression of α2-integrin suppressed FAK-CD-mediated loss of adhesion. Our results suggest a model in which Src regulates adhesion and survival through enhanced expression of the α2-integrin. This provides a mechanism through which Src promotes cellular adhesion and alters the adhesive function of FAK.

Key words: apoptosis, cancer, focal adhesion kinase (FAK), integrin, kinase, Src.

INTRODUCTION

The development of invasive and metastatic cancer requires modulation of adhesion as cells invade their substratum, migrate and then survive in a new environment. These processes require protein kinases, which phosphorylate target proteins on a serine/threonine or tyrosine residue. Focal adhesion kinase (FAK) is a 125 kDa non-receptor tyrosine kinase localized to focal adhesions [1], which are the contact points between cultured cells and their underlying substratum, and are sites of intense tyrosine phosphorylation [2]. FAK is phosphorylated in response to a number of stimuli, including clustering of integrins [3] and plating on fibronectin [4,5] and in response to a number of mitogenic agents[6].

FAK is overexpressed in invasive and metastatic tumours [7], and the *FAK* gene is also amplified in many types of tumours [8], suggesting a role for FAK in adhesion or survival in tumour cells. FAK is associated with a cellular survival signal that is independent of its role in adhesion. FAK transduces a survival signal in response to the extracellular matrix that suppresses a p53-dependent apoptotic response [9]. In tumour cells, attenuation of FAK expression induces detachment and apoptosis, but non-transformed cells are less sensitive [10], suggesting that an FAK-dependent signal is required for tumour cell growth. Furthermore, an activated form of FAK leads to resistance to anoikis [11], and FAK degradation is associated with apoptosis [12,13].

FAK function can be disrupted by overexpression of the FAK C-terminal 360 amino acids. This portion of FAK is produced from a separate transcript in avian cells [14], and this protein [FRNK (FAK-related non-kinase)] [15] inhibits cell spreading and phosphorylation of FAK, the focal adhesion protein paxillin, and to a lesser extent, tensin [16,17]. We have exogenously expressed an analogous fragment of human FAK, which we call FAK-CD (FAK C-terminal domain), and found that FAK-CD causes cell rounding, loss of adhesion and apoptosis in tumour cells, but not in normal cells [18–20]. Other groups have also reported that FAK-CD overexpression does not induce apoptosis in normal cells [21]. Thus FAK-CD provides a convenient means to inactivate FAK function and dissect the signalling requirements for FAK in tumour cells.

FAK was originally identified as a major tyrosine-phosphorylated protein in cells transformed by v-Src, and FAK physically associates with v-Src [22,23]. c-Src is a cytoplasmic tyrosine kinase containing SH2 and SH3 (Src homology 2 and 3) domains, which direct protein–protein interactions at its C-terminus [24]. The v-Src protein contains mutations at the N- and C-termini [25], and an analogous mutation of the C-terminal tyrosine, Tyr^{527}, renders the Src protein transforming in NIH3T3 cells [26]. Similar to FAK, Src is overexpressed in breast cancers, colon cancers and bladder cancers [27–31]. Src can be activated by platelet-derived growth factor receptor [32], the epidermal growth factor receptor, and the c-erb-2/*neu* receptor [33]. Src activation by Neu leads to attachment-independent growth of human breast epithelial cells [34]. One model for Src function is that c-Src activation can bypass the requirement of breast epithelial cells for attachment and integrin signalling, in addition to contributing to cytoskeleton rearrangements and increased migration [35,36].

Abbreviations used: Ad-LacZ, adenovirus expressing *lacZ* gene; FAK, focal adhesion kinase; FAK-CD, FAK C-terminal domain; Ad-FAK-CD, adenovirus expressing FAK-CD; FRNK, FAK-related non-kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; RT, reverse transcriptase; SH2, Src homology 2; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

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Src increases tyrosine phosphorylation of FAK and paxillin [17] and can rescue the detachment of chicken embryo cells from FAK inhibition [17]. The crucial event in this rescue appears to be the phosphorylation of paxillin by Src and the binding of Src to FAK, but the catalytic activity of FAK was not required [17]. For this reason, FAK was postulated to act as a 'switchable adaptor' that recruits c-Src to phosphorylate paxillin. Therefore we speculated whether activated c-Src can rescue the detachment and apoptosis caused by deregulation of FAK in tumour cells. In the present study, we demonstrate that activated Src causes morphological changes in BT474 breast cancer cells, rescues the detachment caused by FAK-CD and leads to decreased apoptosis. Src increases the tyrosine phosphorylation of FAK and paxillin and leads to increased expression of α2-integrin. Exogenous expression of α2-integrin also suppresses loss of adhesion mediated by FAK-CD. These results suggest a novel pathway active in breast tumour cells, namely the regulation of adhesion and survival mediated by the concerted action of Src, FAK and α2-integrin.

EXPERIMENTAL

Cell culture

BT474 and MCF-7 breast ductal carcinoma cells were purchased from the A.T.C.C. (Manassas, VA, U.S.A.). BT474 cells were maintained in RPMI 1640 with 10 % (v/v) foetal bovine serum, 10 μg/ml insulin and 2 mM L-glutamine. MCF-7 cells were cultured in Eagle's minimal essential medium, containing 10 % foetal bovine serum, 10 μg/ml insulin, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. Cells were incubated at 37 °C in a 5 % CO_2 atmosphere.

To prepare stable cell lines expressing activated Src and vector control cells, activated Src (pUSEamp Src-529F; Upstate USA, Charlottesville, VA, U.S.A.) or the host plasmid (pUSEamp; Upstate USA) was transfected into BT474 breast cancer cells, and stable BT474/Src and BT474/vector cell lines were obtained using selection media containing 500 μg/ml Geneticin (Gibco BRL, Gaithersburg, MD, U.S.A.). Src expression was confirmed by Western-blot analysis using an antibody directed to v-Src (Calbiochem, La Jolla, CA, U.S.A.).

BT474 cells expressing α2-integrin were prepared similarly. The α2-integrin coding sequence was excised from the plasmid pSF-α2 (a gift from Dr R. Juliano, University of North Carolina at Chapel Hill, N.C., U.S.A.) using the restriction endonucleases *Xba*I and *Bam*HI, and was cloned into the same sites in the pcDNA3 plasmid (Invitrogen, Carlsbad, CA, U.S.A.), forming the pcDNA3-α2-int plasmid. Cells were transfected with the pcDNA3-α2-int plasmid, and continuously expressing stable cell lines were identified by Western-blot analysis using an anti-α2-integrin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

Immunofluorescence staining, Western-blot analysis and immunoprecipitation

Immunofluorescent staining, Western-blot analysis and immunoprecipitation were performed as described previously [19]. For immunological analyses, the antibodies used were anti-v-Src (Calbiochem), anti-FAK 4.47 (Upstate USA), anti-paxillin (Upstate USA), anti-phosphotyrosine (Upstate USA), anti-FAK pY397 (Biosource, Camarillo, CA, U.S.A.), anti-tubulin (Sigma) and anti-α2-integrin (N-19; Santa Cruz Biotechnology). Actin was stained with Bodipy^{FL}-Phallacidin (Molecular Probes). The pan-Src PP2 inhibitor {4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine} was obtained from Calbiochem [37,38].

Adenoviral transduction

Adenoviruses containing FAK-CD and LacZ were constructed and prepared as described previously [19]. Cells were plated at 1.5×10^6 cells/100 mm culture plate, allowed to attach for 16 h and then infected with Ad-FAK-CD (adenovirus expressing FAK-CD) or Ad-LacZ (adenovirus expressing *lacZ* gene) at an optimal concentration of virus for each cell line. The optimal viral concentration was determined by the infection of breast cancer cell lines with different viral concentrations and the viral titre that produced > 90 % cell infectivity was used. The optimal viral titre was 500 ffu (focus forming units/cell), obtained from the Gene Therapy Center Virus Vector Core (University of North Carolina at Chapel Hill). The optimal viral titre caused protein expression in 100 % of the cells without toxic effect checked by X-Gal (5-bromo-4-chloroindol-3-yl β-D-galactopyranoside) staining for Ad-LacZ transduction and by haemagglutinin immunostaining for Ad-FAK-CD infection, as described previously in [19].

Treatment with the Src-family kinase inhibitor PP2

Cells were starved without serum for 1 h and PP2 was added at 30 μM for 15 min. This optimal PP2 dose was chosen in a dose–response experiment by blocking Src downstream phosphorylation. PP2 was added at 30 μM to the medium for 24 h for Ad-FAK-CD infection experiments.

Detachment assay

After adenoviral infection, the detached cells were collected and counted microscopically on a haemocytometer. The attached cells were harvested by trypsinization and counted. The percentage of detached cells was calculated by dividing the number of detached cells by the total number of cells in three independent experiments.

Apoptosis assay

The detached cells were collected, fixed in 3.7 % (v/v) formaldehyde in 1 × PBS solution and used for the apoptosis assay. Apoptosis was detected by Hoechst 33342 staining or TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay using the ApopTag kit (Oncor, Gaithersburg, MD, U.S.A.) essentially as described in [18,39]. The percentage of apoptosis in detached cells was calculated as the ratio of apoptotic detached cells to the total number of detached cells. The percentage of apoptosis in the total population of cells was calculated as the ratio of apoptotic cells to the total number of cells. Apoptotic rate in attached cells was checked by Hoechst 33 342 staining and was equal to zero. For each experiment, 300 cells/treatment were counted.

PolyHEMA (polyhydroxyethylmethacrylate polymer)-coated tissue culture dishes

Tissue culture dishes were coated with a film of polyHEMA (Aldrich Chemical, Milwaukee, WI, U.S.A.), as described by Valentinis et al. [39]. Briefly, a 12 % solution of polyHEMA in 95 % (v/v) ethanol was mixed overnight, centrifuged at 2500 rev./min to remove undissolved particles and diluted 1:10 with

95 % ethanol. Then, 100 mm dishes were coated with 4 ml of polyHEMA solution and left to dry at room temperature (22 °C). Dishes were washed twice with PBS and once with Hanks balanced salt solution before use.

RT (reverse transcriptase)–PCR analysis

For analysis of integrin mRNA expression, RT–PCR was performed as described in [40]. The oligonucleotide primers for RT–PCR were used as described in [40].

Northern-blot analysis

Total RNA was isolated from breast cancer cell lines using the NucleoSpin II PurificationII Kit according to the manufacturer's instructions (Clontech, Palo Alto, CA, U.S.A.). Purified RNA (10 μg) was separated on denaturing formaldehyde–agarose gels and transferred on to Zeta-Probe membranes (Bio-Rad Laboratories) by the capillary transfer method in 20 × SSC buffer overnight. Prehybridization and hybridization were performed for 1 h at 68 °C in Express Hyb hybridization solution (Clontech) according to the manufacturer's instructions. Northern blots were washed with 2 × SSC and 0.05 % SDS for 30 min at room temperature and with 0.1 % SSC and 0.1 % SDS at 50 °C for 40 min. The blot was exposed to an X-ray (Kodak Biomax MS) film at − 70 °C. After stripping, a ^{32}P-labelled β-actin probe was used for the normalization of the mRNA level.

The hybridization probe for α2-integrin was a 1.8 kb *Eco*RI–*Xba*I fragment isolated from the pcDNA3-α2-integrin plasmid. The purified fragment was labelled with [α-^{32}P]dCTP (Amersham Biosciences, Piscataway, NJ, U.S.A.) by random priming with the High Prime DNA Labelling Kit (Roche Diagnostics, Indianapolis, IN, U.S.A.) and purified using Microspin G-50 columns (Amersham Biosciences). The probe for actin was purchased from Clontech.

Statistical analysis

The statistical significance of differences between the means for samples in each assay was assessed by a Student's *t* test. $P < 0.05$ were considered significant.

FACS analysis of integrin expression

Integrin expression was determined by FACS analysis. Cells were detached with trypsin/EDTA, washed with 1 × PBS/1 % BSA and incubated with an anti-α2-integrin antibody in 1 × PBS/1 % BSA for 1 h on ice. After several washes with PBS, cells were incubated with a secondary FITC-conjugated antibody for 45 min. Cells were washed three times with PBS and fixed in 2 % (w/v) paraformaldehyde in PBS. Cells (10 000/sample) were analysed in FACScan flow cytometer (Becton Dickinson, Mountain View, CA, U.S.A.). Negative controls were set by incubating cells with the secondary antibody alone.

RESULTS

Activated c-Src increases cell spreading and tyrosine phosphorylation of FAK and paxillin in breast cancer cells

We expressed the activated form of Src in BT474 human breast cancer cells, and Src-expressing cells were isolated and confirmed by Western-blot analysis (Figure 1A, right lane). BT474 cells express undetectable levels of Src (Figure 1A, left lane) compared with exogenous Src-mutant-expressing cells (Figure 1A, right lane), and we will refer to the Src-overexpressing cell line as BT474/Src and the control cell line as BT474/vector. Src localized to punctate sites at the cell periphery at the focal adhesions (Figure 1B, lower panel), and Src-expressing cells had a marked increase in cell spreading (Figure 1B).

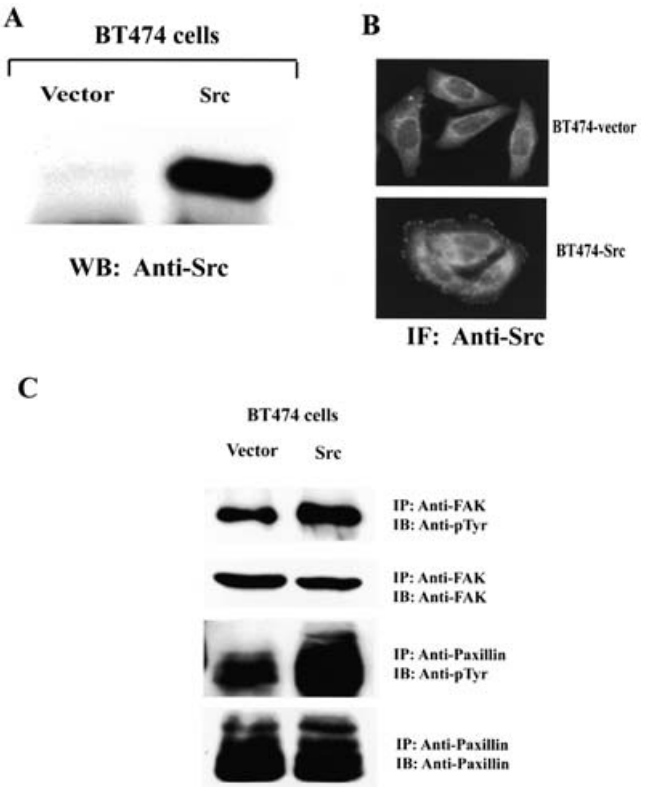

Figure 1 Adhesion regulation by activated Src in BT474 breast cancer cells

(**A**) An activated form of Src was overexpressed in BT474 cells. Cells were transfected with a control plasmid (pUSE; left lane) or pUSE-Src-Y527F (right lane), and expression was verified by Western-blot analysis (WB). (**B**) Src localizes to focal adhesions in BT474/Src cells. BT474/vector (upper panel) and BT474/Src (lower panel) cells were plated on coverslips and stained with an antibody to Src. IF, immunofluorescence staining. (**C**) Increased tyrosine phosphorylation of FAK and paxillin in BT474/Src cells. BT474/vector (left lanes) or BT474/Src (right lanes) cells were lysed, immunoprecipitated (IP) with antibodies to FAK (top two panels) or paxillin (bottom two panels) and subjected to Western-blot analysis (IB) with antibodies to phosphotyrosine (pTyr; first and third panels), FAK (second panel) and paxillin (fourth panel).

Owing to the increased cell spreading in BT474/Src cells, we next examined the expression and phosphorylation state of several downstream targets of Src that mediate cellular adhesion. Tyrosine phosphorylation of FAK and paxillin was increased in BT474/Src cells compared with BT474/vector control cells (Figure 1C, first and third panels), whereas there was no difference in the expression of the two proteins (Figure 1C, second and fourth panels). The increased phosphorylation of FAK (Figure 2, top panel) and paxillin (Figure 2, third panel) was largely decreased by treatment of the cells with PP2 (Figure 2, cf. lanes 3 and 4 of the first and third panels). In contrast, FAK and paxillin phosphorylations were not inhibited by the addition of the control solvent DMSO (Figure 2, first and third panels, lane 3). These results indicate that c-Src regulates the phosphorylation of Paxillin and FAK in breast cancer cells.

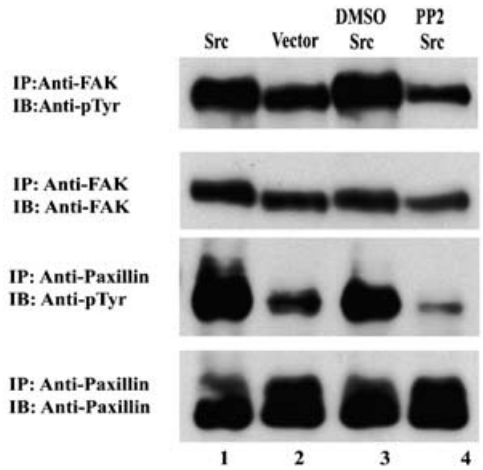

Figure 2 Inhibition of increased tyrosine phosphorylation of FAK and paxillin in BT474/Src cells by PP2

BT474/Src (lanes 1, 3 and 4) or BT474/vector (lanes 2) cells were lysed and immunoprecipitated with antibodies to FAK (top two panels) or paxillin (bottom two panels) and subjected to Western-blot analysis with antibodies to phosphotyrosine (pTyr; first and third panels), FAK (second panel) and paxillin (fourth panel). In lane 3, BT474/Src cells were treated with DMSO alone, whereas in lane 4, BT474/Src cells were treated with the same volume of DMSO and PP2.

Activated c-Src suppresses the loss of adhesion mediated by FAK-CD

Overexpressing the C-terminus of FAK in breast cancer cells causes a significant loss of adhesion and apoptosis [19,20]. We introduced the FAK-CD by adenoviral infection, and most of the infected control cells lost adhesion within 24 h (Figure 3A). BT474/Src cells showed significantly decreased detachment compared with BT474/vector cells after 24 h (Figure 3A, 42 % for BT474/Src cells versus 92 % for BT474/vector cells; $P < 0.05$). Addition of PP2 caused a marked increase in detached BT474/Src cells (Figure 3A, 66 % for Ad-FAK-CD + PP2 cells versus 16 % for control Ad-LacZ + PP2 cells; $P < 0.05$). Thus Src suppresses loss of adhesion mediated by inhibition of FAK.

Activated Src suppresses adhesion-dependent apoptosis in BT474 cells

We have shown previously that inhibition of FAK in BT474 cells has two distinct effects: disruption of cellular adhesion and induction of apoptosis. The two are distinct, since BT474 cells maintained in suspension undergo apoptosis rapidly when treated with FAK-CD [19]. To test whether Src affected the survival function of FAK and whether it was dependent on cellular adhesion, we treated BT474/vector and BT474/Src cells with FAK-CD and harvested detached cells; we then analysed the cells for apoptosis by TUNEL assay or Hoechst staining. There was no significant difference in apoptotic rate between detached BT474/Src cells and BT474/vector control cells after 24 h (67.7 % for BT474/vector versus 52.7 % for BT474/Src; $P = 0.38$) or after 48 h (87.4 % for BT474/vector versus 76.9 % for BT474/Src; $P = 0.28$) after transduction of adenoviral FAK-CD. Next, BT474 cells were maintained in suspension by growing them in dishes coated with polyHEMA, to which the cells cannot attach. Cells were treated with Ad-FAK-CD for 48 h and the percentage of apoptotic cells was determined by TUNEL assay. As before, there was no difference in the apoptotic rate between activated Src cells and vector control cells after transduction of adenoviral Ad-FAK-CD (43.6 % for BT474/Src-Y497F versus 49.6 % for BT474/pUSE; $P = 0.08$). Thus, when cells detach or are in suspension, Src cannot affect the survival function of FAK.

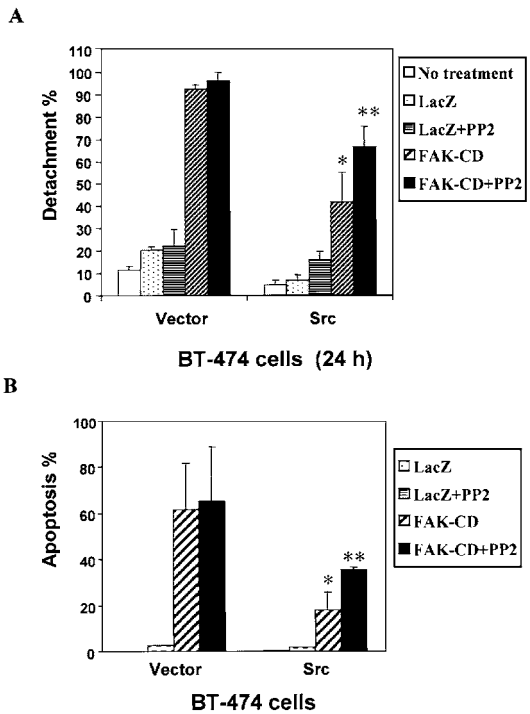

Figure 3 Regulation of adhesion and survival signalling by activated Src in BT474 cells

(**A**) Src suppresses loss of adhesion after treatment with the Ad-FAK-CD adenovirus. The percentage of detached cells 24 h after treatment with the Ad-FAK-CD adenovirus [19] was determined in triplicate. BT474/Src cells had a considerably decreased rate of detachment compared with BT474/vector cells. PP2 reverses the ability of Src to suppress Ad-FAK-CD-mediated loss of adhesion. *$P < 0.03$ for Ad-FAK-CD-treated BT474/Src cells versus Ad-FAK-CD-treated BT474/vector cells; **$P < 0.02$ for Ad-FAK-CD-treated BT474/Src cells with PP2 versus the same without PP2. (**B**) Src suppresses apoptosis in the total population of cells. Apoptotic rate was lower in BT474/Src cells and it was increased by PP2. Apoptosis was determined by TUNEL assay or Hoechst 33342 staining, 24 h after Ad-FAK-CD treatment. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to the total number of cells in three independent experiments in several fields with the fluorescent microscope. For each experiment, 300 cells/treatment were counted. Bars show the means ± S.E.M. for three independent experiments. *$P < 0.04$ for Ad-FAK-CD-treated BT474/Src cells versus Ad-FAK-CD-treated BT474/vector cells; **$P < 0.05$ for Ad-FAK-CD-treated BT474/Src cells with PP2 versus the same without PP2.

However, when apoptosis was analysed in the total population of cells treated with Ad-FAK-CD, the apoptotic rate was significantly low in BT474/Src cells compared with BT474/vector cells (18 % in BT474/Src versus 62 % in BT474/vector cells; $P = 0.03$) and was increased to > 35 % ($P < 0.05$) by PP2 (Figure 3B).

Our results suggested that Src functions as an adhesion-dependent activator of FAK. To test this, we treated cells with Ad-FAK-CD and compared BT474/vector and BT474/Src cells that were adherent or non-adherent for levels of FAK tyrosine phosphorylation. As expected, adherent BT474/Src cells had increased levels of FAK and paxillin phosphorylation (Figure 4, lanes 1 and 2). In detached cells, both BT474/Src and BT474/vector cells lost FAK and paxillin tyrosine phosphorylation (Figure 4, first and third panels, lanes 3 and 4 respectively). Thus Src phosphorylation of FAK and paxillin is part of an adhesion-dependent signalling cascade.

We next examined the effect of activated Src on FAK localization after infection with Ad-FAK-CD. FAK normally localizes

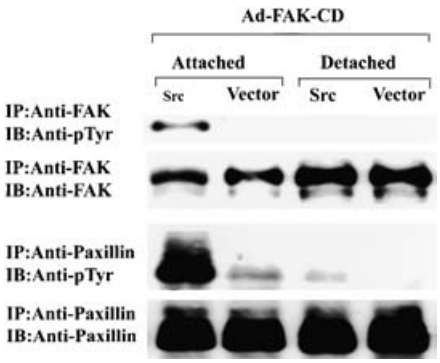

Figure 4 Adhesion-dependent Src-mediated phosphorylation of FAK and paxillin

BT474/Src (lanes 1 and 3) and BT474/vector (lanes 2 and 4) cells were infected with Ad-FAK-CD, and the attached (lanes 1 and 2) and detached (lanes 3 and 4) cells were harvested. Cells were lysed and proteins were immunoprecipitated (IP) with antibodies to FAK (top two panels) or paxillin (bottom two panels) and subjected to Western-blot analysis with antibodies to phosphotyrosine (first and third panels), FAK (second panel) and paxillin (fourth panel). The Src-mediated increase in the phosphorylation of FAK and paxillin tyrosine (first and third panels, cf. lanes 1 and 2) was not detected in detached cells.

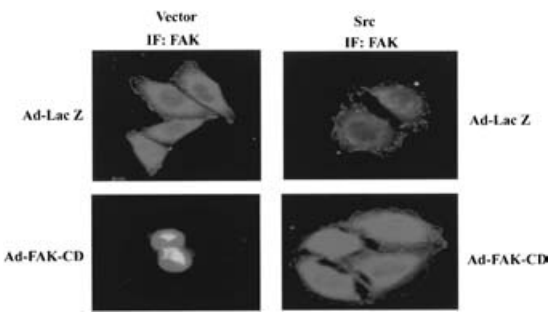

Figure 5 Src-mediated localization of FAK after infection with Ad-FAK-CD

BT474/vector and BT474/Src cells were infected with Ad-FAK-CD and control Ad-LacZ adenoviruses and immunostained with antibodies to FAK. Representative fields are shown. Most of the BT474/vector cells had rounded morphology and were detached (Figure 3A), and FAK was displaced from focal adhesions after Ad-FAK-CD infection. In contrast, BT474/Src cells expressed increased attachment (Figure 3A), and FAK localized at focal adhesions after Ad-FAK-CD treatment.

to punctate focal adhesion sites at the cell periphery, and after infection with Ad-FAK-CD, FAK is displaced from these sites in BT474 vector cells versus Ad-LacZ control cells (Figure 5, left panels). In BT474/Src cells treated with Ad-FAK-CD, FAK remained at focal adhesions (Figure 5, right panels). Thus Src regulates the localization of FAK after infection with the Ad-FAK-CD adenovirus in BT474 cells.

Activated Src causes enhanced adhesion and suppressed apoptosis in MCF-7 cells

Breast cancer cell lines are highly heterogeneous, and owing to increased genome instability in cancer cells, it is preferable to analyse cellular phenotypes in different cell lines. We transfected the pUSE control plasmid or pUSE-Src-Y527F into MCF-7 breast cancer cells, established stable transfectants and verified overexpression of Src by Western-blot analysis (Figure 6A, upper panel), with vinculin as a control for loading (Figure 6A, lower panel). Similar to BT474 cells, MCF-7/Src cells exhibited increased phosphorylation of FAK and paxillin (Figure 6B, first and third panels) and a flattened morphology (Figure 6C, top panels).

The activated, Tyr^{397}-phosphorylated form of FAK was increased in MCF-7/Src cells (Figure 6C, left and right panels). Actin was stained in MCF-7/vector and MCF-7/Src cells (Figure 6C, top panels) as was the Tyr^{397}-phosphorylated form of FAK (Figure 6C, middle panels). Activated FAK co-localized with actin at focal adhesions when images were merged (Figure 6C, bottom panels).

The activated, Tyr^{397}-phosphorylated form of FAK was increased in MCF-7/Src cells (Figure 7A, lanes 1 and 2). Src overexpression resulted in increased activity of FAK in attached cells infected with Ad-FAK-CD (Figure 7A, lane 4) compared with BT474/vector cells (Figure 7A, lane 3). When detached, both cell lines infected with Ad-FAK-CD expressed inactive FAK (Figure 7A).

As for BT474 cells, Src overexpression decreased loss of adhesion after infection with the FAK-CD-expressing adenovirus (Figure 7B). Similar to BT474 cells, the adhesive effects of Src were reversed with PP2 (Figure 7B), whereas PP2 had minimal effect on detachment in the control cells infected with Ad-LacZ (Figure 7B). In detached cells, Src was not capable of affecting the survival of MCF-7/Src cells infected with Ad-FAK-CD, as in BT474/Src cells. In contrast, Ad-FAK-CD-induced apoptosis was suppressed in the total population of MCF-7/Src cells compared with MCF-7/vector cells, and the apoptotic rate was significantly increased by PP2 (Figure 7C). Thus the ability of Src to protect cells from loss of adhesion and adhesion-dependent apoptosis after FAK inhibition is not specific to BT474/Src cells, but was also observed in another cell line, MCF-7/Src.

Src overexpression leads to increased α2-integrin expression

One potential mechanism for the increased adhesion in c-Src-Y527F-overexpressing breast cancer cells is the increased expression of cellular adhesion molecules. For this reason, we analysed the expression of six different integrins in BT474/vector and BT474/Src cells by RT–PCR. BT474/Src cells had significantly increased expression of α2-integrin, whereas the expression levels of α1-, α5-, αV-, β1- or β3-integrins were not affected (Figure 8A). Increased α2-integrin expression was confirmed by two additional methods, namely Northern-blot analysis in both BT474 and MCF-7 cells (Figure 8B) and Western-blot analysis (Figure 8C). We performed FACS analysis to examine whether α2-integrin is expressed on the cell surface (Figure 8D). Src-overexpressing cells increased cell-surface expression of α2-integrin compared with control vector cells (Figure 8D). The increased α2-integrin expression was significantly decreased in both BT474/Src and MCF-7/Src cells by PP2 (Figure 8B, lanes 3 and 7), whereas the PP2 solvent DMSO had no inhibitory effect (Figure 8B, lanes 4 and 8). Thus, in two different breast cancer cell lines, Src overexpression led to increased α2-integrin expression at the RNA and protein levels.

To test the effects of α2-integrin overexpression on FAK-mediated adhesion, we transfected BT474 cells with the plasmid pcDNA3-α2-int and selected stably expressing clones. The cell line that stably overexpressed the α2-integrin protein was identified by Western-blot analysis (Figure 9A, lane 1). By immunostaining analysis, we observed overexpression of α2-integrin on the cell surface in BT474/α2-int cells compared with control vector cells (Figure 9B). BT474/α2-int cells had a decreased rate of detachment on exposure to the FAK-CD adenovirus (41.4 % versus 91.7 % in BT474/vector cells; $P < 0.01$; Figure 9C). Thus α2-integrin overexpression leads to resistance to loss of adhesion after infection with the FAK-CD adenovirus. These experiments

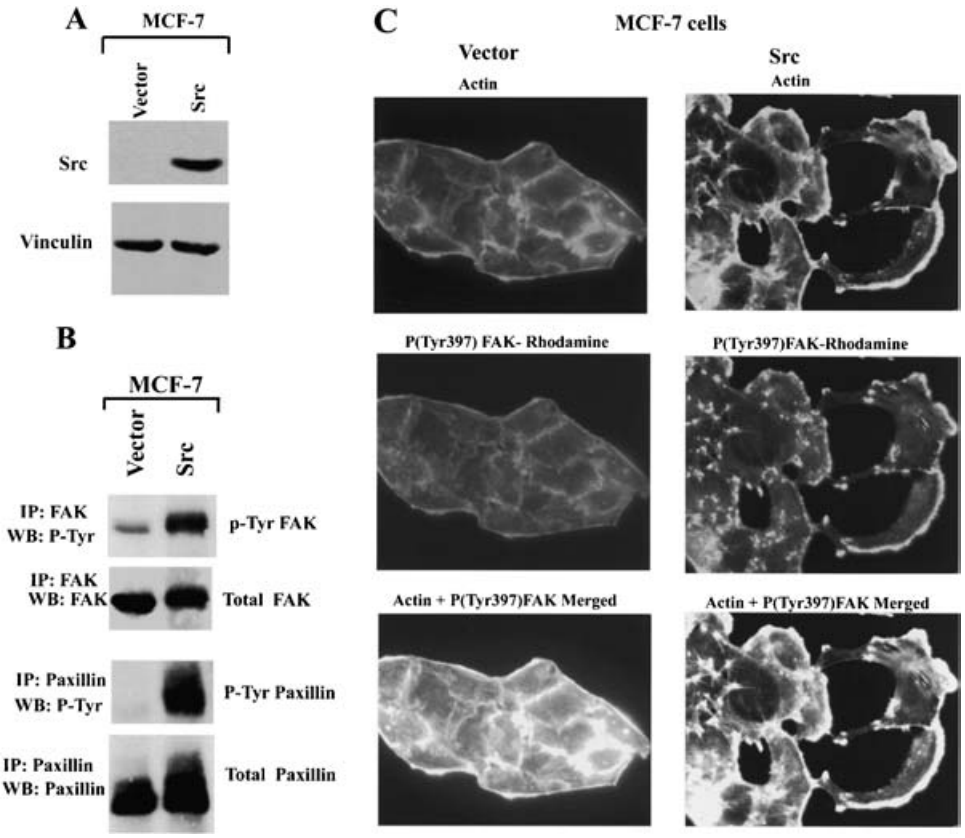

Figure 6 Increased phosphorylation of FAK and paxillin and enhanced cellular adhesion in MCF-7/Src cells

(**A**) MCF-7 breast cancer cells were transfected with the plasmid pUSE (vector) or pUSE-Src-Y497F (Src). Expression of Src was analysed with a monoclonal antibody to Src (upper panel), with vinculin as a control for loading (lower panel). (**B**) Increased tyrosine phosphorylation of FAK and paxillin in MCF-7/Src cells. MCF-7/vector (left lanes) or MCF-7/Src (right lanes) were lysed and immunoprecipitated with antibodies to FAK (top two panels) or paxillin (bottom two panels) and subjected to Western-blot analysis with antibodies to phosphotyrosine (first and third panels), FAK (second panel) and paxillin (fourth panel). (**C**) Activated FAK localizes to focal adhesions in MCF-7/Src cells. MCF-7/vector (left panels) and MCF-7/Src (right panels) cells were stained for actin (top panels) or Tyr^{397}-phosphorylated FAK (middle panels) and the staining patterns were merged (bottom panels). MCF-7/Src cells had increased activation of FAK (Tyr^{397}-phosphorylated FAK) versus MCF-7/vector cells. Whereas activated FAK stained in an evenly distributed pattern in MCF-7/vector cells (bottom left panel), FAK localized to brightly clustered focal adhesion sites (co-localized with actin) in MCF-7/Src cells (bottom right panel).

provide evidence that $\alpha 2$-integrin contributes to pro-adhesive effects in Src-overexpressing cells.

DISCUSSION

FAK expression is increased in a variety of human tumours, and inhibition of FAK leads to loss of adhesion and apoptosis that is specific to tumour cells. However, the adhesion signalling pathways affected by FAK in tumours are poorly understood. In addition, it was not clear whether FAK-mediated survival signalling occurred through the same pathway through which FAK regulated adhesion in tumours. Our results clearly demonstrate that FAK-mediated survival signalling is dependent on adhesion in Src-overexpressing breast cancer cell lines, and that Src enhances the adhesive function by increasing the expression of adhesive $\alpha 2$-integrin. We had shown previously that inhibition of FAK leads to apoptosis even under conditions in which cells were deprived of adhesion [19]. In the present study, we have developed a system in which the adhesive function of FAK is hyperactivated, and the anti-apoptotic function in detached cells is unaffected, but in the total population of cells it suppressed apoptosis. The Ad-FAK-CD-induced apoptosis was decreased by overexpression of activated Src and hyperactivation of FAK owing to increased adhesion. The increased tyrosine phosphorylation of FAK and paxillin and the increased $\alpha 2$-integrin expression may function in stabilizing the focal adhesions and localization of FAK at focal adhesion sites. Furthermore, the modulation of $\alpha 2$-integrin expression by the Src inhibitor PP2 suggests a mechanism for the role of Src in regulating integrin-dependent focal adhesion. Interestingly, MCF-7/Src cells were more sensitive to FAK-CD + PP2-induced apoptosis compared with BT474/Src cells. This may be explained by the more robust Src-dependent downstream signalling in MCF-7/Src cells compared with BT474/Src cells and the complete abrogation of $\alpha 2$-integrin expression by PP2 in MCF-7/Src cells. Alternatively, there may be other cell-type-specific mechanisms to explain the difference. These results are similar to the findings of Windham et al. [41], demonstrating that enhanced expression of activated Src increases resistance to anoikis in human colon tumour cell lines.

We have provided a potential mechanism through which activated Src leads to increased adhesion and the increased transcription of $\alpha 2$-integrin. Furthermore, $\alpha 2$-integrin expression was capable of suppressing the loss of adhesion caused by inhibition of FAK function. $\alpha 2$-Integrin has been linked to increased migration and adhesion in breast cancer cells [42] in a manner that requires the p38 MAPK (mitogen-activated protein kinase) [43]. $\alpha 2$-Integrin contributes to robust activation of intracellular signalling [44] and cell-cycle progression [45]. Thus enhanced adhesion is consistent with known phenotypes associated with

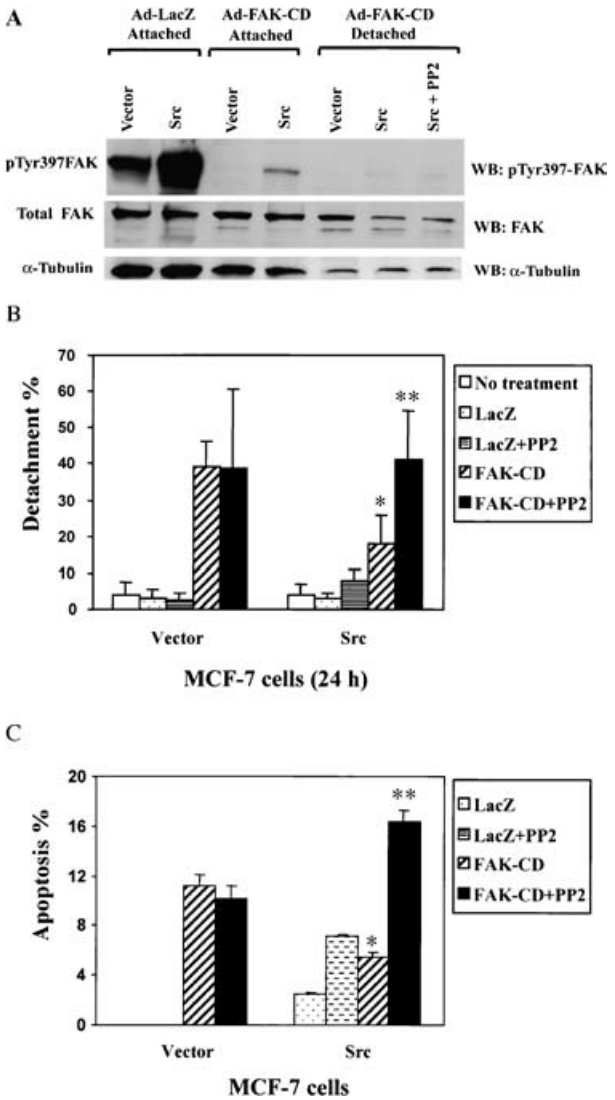

Figure 7 Loss of FAK phosphorylation after detachment in Ad-FAK-CD-treated MCF-7 cells

(**A**) MCF-7/vector (lanes 1, 3, 5 and 7 from the left) and MCF-7/Src (lanes 2, 4 and 6 from the left) cells were treated with Ad-LacZ (lanes 1 and 2) or Ad-FAK-CD (lanes 3–7), and FAK Tyr^{397} phosphorylation (top panel), FAK expression (middle panel) and tubulin expression (bottom panel) were determined by Western-blot analysis. FAK phosphorylation was high in attached MCF-7/vector and MCF-7/Src cells (lanes 1 and 2), but was dramatically decreased in cells that lost adhesion (lanes 3–7). (**B**) Src increases adhesion and adhesion-dependent survival in MCF-7/Src cells. Adhesion of MCF-7/Src and MCF-7/vector cells infected with Ad-FAK-CD and control Ad-LacZ was analysed as in Figure 3(C). Activated Src caused increased adhesion of MCF-7/Src cells after Ad-FAK-CD infection compared with control vector cells ($*P < 0.05$ for MCF-7/Src + Ad-FAK-CD versus MCF-7/vector + Ad-FAK-CD), reversed by PP2 ($**P < 0.05$ for MCF-7/Src + Ad-FAK-CD + PP2 versus MCF-7/Src + Ad-LacZ + PP2). (**C**) Activated Src causes inhibition of apoptosis in the total population of MCF-7/Src cells. Apoptosis was analysed as in Figure 3(B). Apoptosis is suppressed in MCF-7/Src cells compared with MCF-7/vector cells, and is reversed by PP2. $*P < 0.02$ for Ad-FAK-CD-treated MCF-7/Src cells versus Ad-LacZ-treated MCF-7/vector cells; $**P < 0.005$ for Ad-FAK-CD-treated MCF-7/Src cells with PP2 versus the same without PP2. Bars show means ± S.E.M. for three independent experiments.

increased expression of α2-integrin. However, we note that in some non-tumourigenic breast cell lines [46], Erb-B2 (erythroblastic leukaemia viral oncogene homologue 2) leads to decreased α2-integrin expression, indicating that the pathways regulating α2-integrin expression are complex and may vary between cell lines.

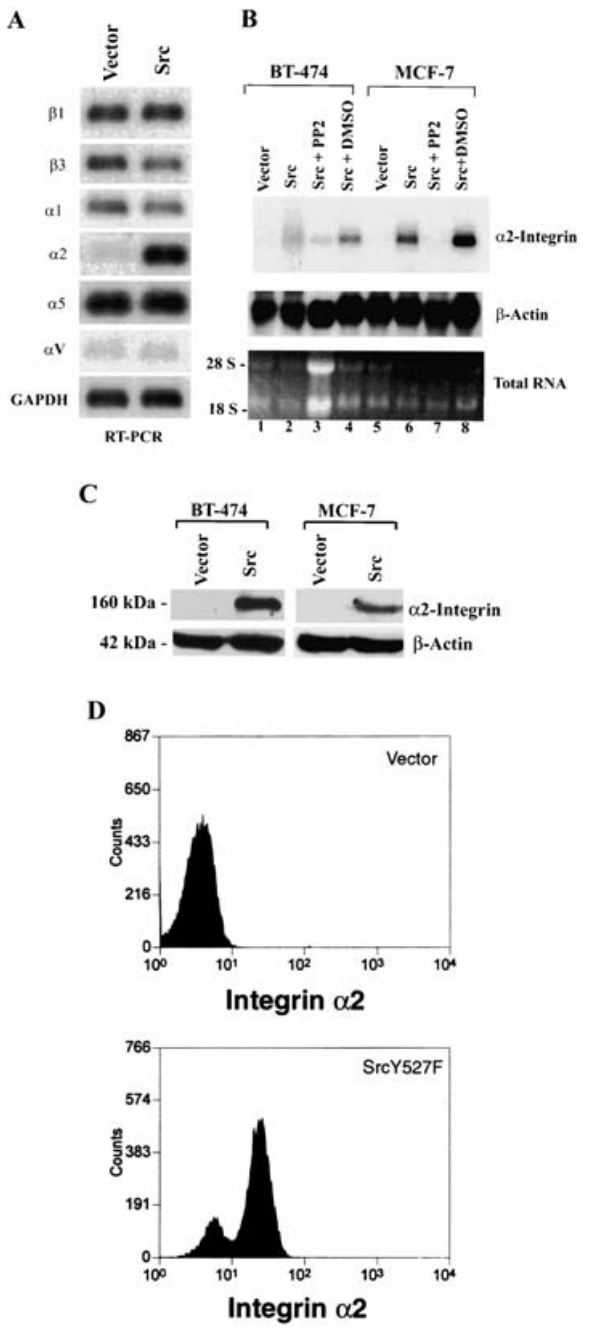

Figure 8 Increased α2-integrin expression by activated Src

(**A**) RT–PCR of cDNA from BT474/vector (left lanes) and BT474/Src (right lanes) cells amplified with primers to the indicated integrins (top six panels) or glyceraldehyde-3-phosphate dehydrogenase (bottom panel) as a loading control. BT474/Src cells had highly increased expression of α2-integrin (fourth panel). (**B**) Northern-blot analysis of α2-integrin expression. RNA was purified from the indicated cell lines and visualized with ethidium bromide (bottom panel) or transferred on to nylon membranes and probed for β-actin (middle panel) or α2-integrin (top panel). In lanes 3 and 7, cells were treated with PP2, whereas cells in lanes 4 and 8 were treated with an equal concentration of the PP2 carrier DMSO. Activated Src led to increased transcription of α2-integrin that was reversed with PP2. (**C**) The α2-integrin protein is increased in Src-expressing breast cancer cells. The indicated cell lines were lysed and subjected to Western-blot analysis with an antibody to α2-integrin (upper panel) or β-actin (lower panel). (**D**) FACS analysis of cell-surface α2-integrin expression in BT474/vector and BT474/Src cells. The *y*-axis represents cell number and the logarithmic *x*-axis represents FITC-fluorescence intensity in arbitrary units and corresponds to the α2-integrin expression on the cell surface. The expression of α2-integrin was increased in BT474/Src cells compared with BT474/vector cells. Experiments were repeated three times with the same result and the representative result is shown.

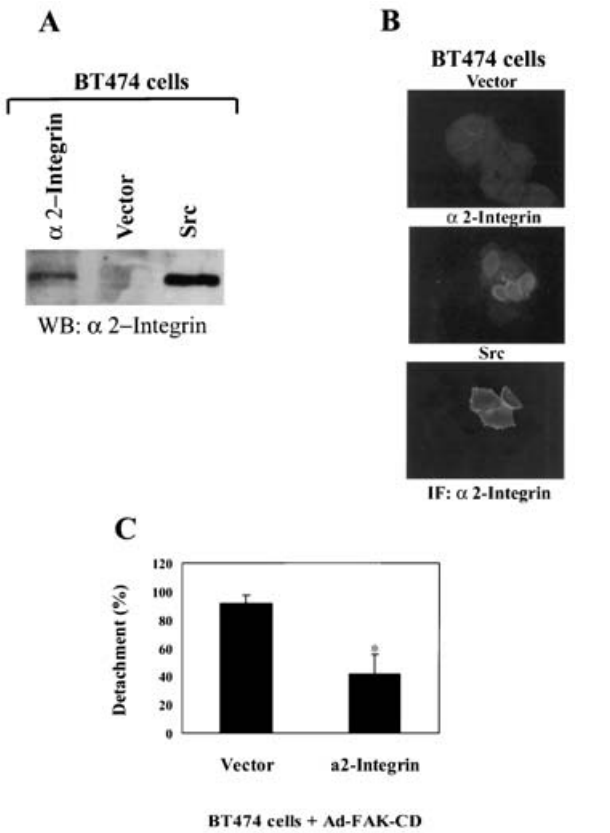

Figure 9 Inhibition of detachment by α2-integrin overexpression in Ad-FAK-CD-infected BT474 cells

(**A**) α2-Integrin was overexpressed in BT474 cells. BT474 cells transfected with plasmids directing expression of α2-integrin (left lane), a control plasmid (middle lane) or an Src-expressing plasmid were subjected to Western-blot analysis for α2-integrin. Expression of α2-integrin-transfected cells was increased in α2-integrin-transfected cells (left lane) and BT474/Src cells (right lane). (**B**) Overexpression of α2-integrin on the cell surface in α2-integrin-transfected BT474 and BT474/Src cells. Immunofluorescence staining (IF) was performed with an anti-α2-integrin monoclonal antibody and an FITC-conjugated secondary antibody. The images are representative of three separate experiments. (**C**) α2-Integrin expression rescues detachment in Ad-FAK-CD-treated cells. BT474/vector (left bar) and BT474/α2-integrin (right bar) cells were infected with the Ad-FAK-CD adenovirus, and detached cells were counted in triplicate. Detachment of Ad-FAK-CD-treated BT474/α2-integrin cells was significantly decreased compared with BT474/vector cells ($^*P < 0.008$). Error bars indicate S.D. The results are representative of three independent experiments.

Src kinase activated α2-integrin expression most probably through increased phosphorylation of transcription factors. Some of the transcription factor-binding sites (AP-1, AP-2 and six SP-1 sites) for the gene encoding α2-integrin are characterized [47] and the upstream regulators of α2-integrin expression are known in some detail. In fibroblasts, platelet-derived growth factor drives the expression of α2-integrin mRNA, and its promoter induction requires the function of protein kinase C-ζ, which indicates that phosphorylation events are required for promoter-directed transcription of α2-integrin [48]. Expression of α2-integrin is also activated by nuclear factor κB through an indirect mechanism [49]. Interestingly, Src activation has been linked to nuclear factor κB activation [50–53], suggesting a mechanism through which activated Src could enhance the expression of α2-integrin.

We have shown that FAK is overexpressed in a number of tumour types, including breast cancer [54–57], and this finding has been confirmed by other groups in different tumour systems [8,58]. Src is also overexpressed in a number of tumour types, including breast and colon cancers [41,59]. Thus Src and FAK can co-operate to regulate adhesion and survival in tumours. In the present study, we have shown that overexpression of Src led to increased α2-integrin, which is reversed by PP2. This is the first step to study further the Src-dependent regulation of integrin expression leading to mediation of downstream survival signalling. The present study shows a novel mechanism of adhesion-dependent survival in breast cancer cell lines.

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