Role of c-Src in Human MCF7 Breast Cancer Cell Tumorigenesis

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To study the role of c-Src in breast cancer tumorigenesis, we generated a cell line derived from MCF7 carrying an inducible dominant negative c-Src (c-SrcDN: K295M/Y527F) under tetracycline control (Tet-On system). c-SrcDN expression caused phenotypic changes, relocation of c-Src, Fak, and paxillin, and loss of correct actin fiber assembly. These alterations were coupled to increased Fak-Tyr397 autophosphorylation and to inhibition of Fak-Tyr925, p130CAS, and paxillin phosphorylation. An increased association of total Src with Fak with Fak and a decreased interaction of p130CAS and p85-P13K with Fak were also observed. SrcDN inhibited cell attachment, spreading, and migration. Serum and EGF-induced stimulation of cell proliferation and Akt phosphorylation were also significantly reduced by SrcDN, whereas p27kip1 expression was increased. Consistently, silencing c-Src expression by siRNA in MCF7 cells significantly reduced cell migration, attachment, spreading and proliferation. Inoculation of MCF7 cells carrying inducible SrcDN to nude mice generated tumors. However, doxycycline administration to mice significantly reduced tumorigenesis, and when doxycycline treatment was installed after tumor development, a significant tumor regression was observed. In both situations, inhibition of tumorigenesis was associated with decreased Ki67 staining and increased apoptosis in tumors. These data undoubtedly demonstrate the relevance of the Src/Fak complex in breast cancer tumorigenesis.

The Src family of non-receptor protein tyrosine kinases plays critical roles in a variety of cellular signal transduction pathways that are involved in cell division, motility, adhesion, angiogenesis, and survival (1).

Studies, mostly performed in colon cancer cells and fibroblasts, revealed that the oncogenic potential of increased Src activity in tumor cells is pleiotropic involving control of cell proliferation and the regulation of cytoskeletal-linked events, such as ECM-adhesion, migration, spreading, and invasion (2–4). These biological effects are associated to Src substrates, among them, Fak, p130CAS and paxillin, which are involved in integrin signaling and in adhesion dynamics (1, 5). However, in breast cancer cells, the relevance and dispensability of each member of this complex is not fully understood, and it is still intriguing the importance of their kinase activity and their role as adaptor proteins to control the signaling pathways they trigger. A recent work in mouse embryo fibroblasts showed that Fak is dispensable for v-Src-induced transformation but would exert either positive or negative effects on signaling or motility depending on the pathways activated (6).

The Src family of tyrosine kinases has a modular prototypic structure with a membrane-targeting signal, which is always myristoylated and sometimes palmitoylated, and Src homology domains SH2 and SH3, which facilitate protein-protein interactions. The kinase domain contains the ATP binding site Lys295 (chicken nomenclature) and an autophosphorylation tyrosine residue (Tyr416). The carboxyl tail contains Tyr527, which regulates Src kinase activity. Phosphorylation of Tyr527 by CSK, favors intramolecular interaction with the SH2 domain, inducing an inactive conformation of the Src kinases (1, 7). There are data in vivo and in tissue culture supporting the fact that Src kinases exert their biological actions not only through their enzymatic activity but also through their capacity to interact with other proteins via the SH2 and SH3 domains, generating complexes of signaling molecules involved in diverse signal transduction pathways (4, 8–10). The combined mutations in the ATP binding site (K295M) and the regulatory C-terminal tyrosine residue (Y527F) generate a c-SrcDN mutant (K295M/Y527F) with an open conformation in which SH2 and SH3 domains may interact with other proteins but devoid of enzymatic activity; therefore, SrcDN is considered a dominant negative mutant of c-Src. Its expression was more efficient than c-Src-K-(K295M) in inhibiting prolactin-medi-
Src and Breast Cancer Tumorigenesis

aded proliferation of W53 lymphoid cells, by competing with endogenous activated Fyn and Lyn Src kinases (8).

c-Src is frequently overexpressed and/or aberrantly activated in a variety of epithelial cancers, including breast cancer (11–15). In addition, c-Src is associated with and activated by a variety of growth factor receptors involved in the growth and survival of human breast cancer cells (16–22). Also, c-Src tyrosine kinase activity appears to be required for PyV middle T antigen-induced mammary tumorigenesis (23). Although these data support the importance of c-Src in breast cancer, the direct effect of c-Src functional inactivation on the transforming phenotype and tumorigenic potential of breast cancer cells has not been yet directly examined. Therefore, we generated MCF7 human breast cancer cells expressing SrcDN under the control of tetracycline (Tet-On system) in order to discriminate the specific role of the Fak and c-Src kinases, and to overcoming the drawback of Src inhibitors that could affect other signaling molecules (24, 25). In MCF7 breast cancer cells, we observed that induction of SrcDN expression altered cell morphology, and caused a significant impairment of cell migration, adhesion, and spreading. These effects were associated with the delocalization and reduced tyrosine phosphorylation of critical proteins involved in integrin signaling and adhesion dynamics, such as Fak (Tyr925), p130Cas and paxillin. Expression of SrcDN also, significantly reduced cell proliferation in vitro, and led to a clear decrease in Akt phosphorylation and to a higher expression of the cell cycle inhibitor p27kip1. Depletion of endogenous c-Src levels by means of siRNA had similar effects, showing the relevance of c-Src in breast cancer cells migration, adhesion, spreading and proliferation. Finally, expression of c-SrcDN reduced tumor growth in nude mice and more importantly, it was able to induce tumor regression. The antitumorigenic ability of induced SrcDN was associated with decreased proliferation, induction of apoptosis and reduction of angiogenic potential. Together these results provide insight on the mechanisms of the Src-Fak complex in the control of breast cancer cells proliferation, migration, spreading and survival, and the relevance of c-Src for the correct performance of this complex, pointing to this signaling protein as a potential target for therapeutic agents.

EXPERIMENTAL PROCEDURES

Reagents—Tet system approved fetal bovine serum was from BD Biosciences Clontech (Palo Alto, CA). The BCA protein assay reagent was from Pierce. Mouse mAb 327 against c-Src was a gift from J. S. Brugge (Harvard University, Boston, MA). Anti-avian Src mouse mAb clone EC10, anti-phosphotyrosine mAb 4G10, and anti-p-Fak (Tyr996) were purchased from UBI (Lake Placid, NY), and anti-p-Fak (Tyr925) was from US Biological (Swampscott, MA). Affinity-purified rabbit polyclonal antibodies to Src kinases (SRC-2), Fak (A17), Erk2 (C-14), VEGF (A-20), and Akt (H-136) were from Cell Signaling (Beverly, MA). Anti-p27 and anti-p53 were from BD Biosciences PharMingen (San Diego, CA), polyclonal antibody to p85-p130 was from Upstate (Charlottesville, VA) and mAb anti-α-tubulin was from Sigma. mAb anti-poly (ADP-ribose) polymerase (PARP) was from Biomol (Biomolecules for Research Success, Plymouth Meeting, PA). Secondary horseradish peroxidase-conjugated antibodies were purchased from BIOSOURCE International (Camarillo, CA). FITC-conjugated rabbit anti-mouse IgG antibody was obtained from Dako (Dakocytomation, Glostrup, Denmark). The enhanced chemiluminescence (ECL) kit was from GE Amersham Biosciences. BCA protein assay was from Pierce. Trypan blue, crystal violet, doxycycline (Dox), puromycin, phallolidin, protein G-Sepharose, and protein A-Sepharose were purchased from Sigma-Aldrich. mAb anti-poly (ADP-ribose) polymerase (PARP) was from Dako (Dakocytomation, Glostrup, Denmark). The enhanced chemiluminescence (ECL) kit was from GE Amersham Biosciences. BCA protein assay was from Pierce. Trypan blue, crystal violet, doxycycline (Dox), puromycin, phallolidin, protein G-Sepharose, and protein A-Sepharose were purchased from Sigma-Aldrich. G418 sulfate (Geneticin) was from invitrogen. Acrylamide/bis 40% solution, 29:1 (3.3% C) and ammonium persulfate were obtained from Bio-Rad.

Generation of MCF7 Human Breast Cancer Cells Bearing a Doxycycline-inducible SrcDN—Clone 89rtTA expressing the rTAT (the reverse tetracycline transactivator) was obtained by transfection of MCF7 cells with pHUD17–neo as previously described (26). Clone 89rtTA cultured in complete medium (DMEM supplemented with 5% Tet-free FCS, 2 mm glutamine, 100 international units/ml penicillin, and 100 μg/ml streptomycin), was maintained with 0.2 mg/ml G418. Cells were then cotransfected with SrcDN (avian c-Src K295M/Y527F) (8) cloned into pTET-Splice (Invitrogen) and pBabe-Puromycin by calcium phosphate method. Control cells were cotransfected with pTET-Splice (Invitrogen) and pBabe-Puromycin. Transfected cells were then selected with 2 μg/ml puromycin. The resistant clones were expanded and tested for the induction of SrcDN expression upon addition of 2 μg/ml Doxy by Western blotting with mAb EC10 to detect avian SrcDN. Four clones with very low basal SrcDN expression in the absence of Doxy, and high induction levels with Doxy were selected (1, 2, 29, and 30). For further experiments these clones were maintained in complete medium containing 0.2 mg/ml of G418 and 0.5 μg/ml puromycin.

Generation of MCF7 Cells Expressing c-Src-siRNA—Duplexes for hairpin siRNA expression contained the unique 19-nucleotide sequence (AAACTCCCCCTTGCTCATGTAC) that specifically targets the kinase domain of human c-Src (BLAST, NCBI Genome), in both sense and antisense orientation, separated by a 9-nucleotide (TTCAAGAGA) spacer sequence. The 5′-end corresponds to the BglI site, while the 3′-end contains the T5 sequence and HindIII corresponding nucleotides. The bacterial EGFP sequence was used as control (27). Forward and reverse oligonucleotides were annealed to produce the dsRNA, digested with BglII and HindIII and cloned into pSUPERIOR.puro (OligoEngine Inc., Seattle, WA), and correct insertion and insert sequence checked by sequencing. Stable transfection of MCF7 cells with the plasmids containing the siRNA for human c-Src or EGFP was carried out using jetPEYTM (Polyplus Transfection, Illkirch, France), according to the manufacturer’s instructions. Clones were selected with puromycin (1 μg/ml) and tested for c-Src expression by Western blotting using 327 mAb.

Cell Proliferation Assay—Cell proliferation was determined by staining the cells with crystal violet. Cells were plated at a density of 10⁵ cells per well (24-well-plates) with complete medium in the presence or absence of Doxy. The next day medium was replaced for phenol red-free and serum-free medium.
DMEM supplemented with 10% serum or with EGF (100 ng/ml), in the presence or absence of 2 µg/ml Doxy; 72 h later the number of cells per well was determined by crystal violet assay. Briefly, following cell fixation with glutaraldehyde 1%, cells were stained with crystal violet 1% and adhesion was quantified by washing the cells, eluting the dye with 1 ml/well of 10% acetic acid and measuring the absorbance at 600 nm. Cell proliferation was also determined by counting viable cells with trypan blue exclusion dye, as follows: cells were seeded (5 × 10^5/60-mm plate) in complete medium in the presence or absence of Doxy. After 16 h (t = 0), the culture medium was changed for phenol red-free DMEM supplemented with 10% serum or with EGF (100 ng/ml), in the absence or presence of 2 µg/ml Doxy, and 72 h later adherent cells were detached mixed with a 0.4% trypan blue solution (1:1) and loaded on a hemocytometer. The percentage of viable cells was calculated to determine cell growth.

**Bromodeoxyuridine (BrdU) Pulse-label Experiments and Flow Cytometry Analysis**—Cell cycle was analyzed by the BrdU/anti-BrdU method. Briefly, cells were plated (1.5 × 10^6/90-mm plate) in serum-supplemented medium in the presence or absence of Doxy (2 µg/ml) and incubated for 48 h. Afterward, cells were pulse-labeled for 30 min with 10 µM of BrdU, washed twice with culture medium and incubated in fresh serum supplemented medium in the presence or absence of Doxy for different time periods. At given times, cells were detached labeled with fluorescein isothiocyanate (FITC)-conjugated-anti-BrdU (Becton Dickinson, San Jose, CA) and propidium iodide, and analyzed as previously described (8).

**Cell Migration Assay**—Cells were seeded at 5 × 10^5/60 mm, and grown in complete medium for 16 h. Then cells were incubated in the presence or absence of Doxy (2 µg/ml) for 24 h to allow expression of SrcDN mutant. Subsequently, the cell monolayers were scarred with a sterile micropipette tip and incubated for another 24 h with or without Doxy. For each sample three defined areas were monitored during this period. The photographs were taken at the beginning of the assay (t = 0 h) and 24 h later (t = 24 h). Magnification, ×100.

**Cell Adhesion and Spreading Assays**—Cell adhesion and spreading assays were essentially performed as described previously (28). Briefly, 2 × 10^5 cells, pretreated with or without Doxy for 48 h, were seeded in 96-well plates precoated with fibronectin (1 mg/ml) in serum-free DMEM in presence or absence of Doxy. Control cells were seeded in bovine serum albumin (1 mg/ml) precoated wells. After 2 h of incubation at 37 °C cells were photographed (magnification, ×400) to determine cell spreading. Cell adhesion was quantified by washing twice with PBS to remove non-adherent cells, fixing the adhered cells and staining with crystal violet 1% and quantifying, as described previously (see cell proliferation assays). Spreading and adhesion were also determined in plastic wells. A similar method was used but with 2 × 10^5 cells, pretreated with or without Doxy for 48 h. Cell spreading was quantified by counting round and spread cells in at least six fields (~200 cells per field) per sample.

**Immunoprecipitation and Western Blot Analysis**—Cells (1 × 10^6 cells/dish) were washed twice in ice-cold TBS (20 mM Tris-HCl, pH 7.6, 140 mM NaCl) with 0.1 mM Na_3VO_4 and lysed at 4 °C in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 30 mM sodium pyrophosphate, 5 mM EDTA, 0.5% Nonidet P40, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM iodoacetamide, 1 mM ortho-phenanthroline). Cell lysates were processed for immunoprecipitation and Western blot, as previously described (8).

**Immunofluorescence Microscopy and Confocal Analysis**—Cells were plated at 1 × 10^4 per chamber slide well, fixed, and permeabilized in 100% methanol for 10 min at −20 °C (anti-Src 327, anti-Fak, anti-paxillin), or fixed with 2% formaldehyde in PBS and permeabilized in PBS-T (PBS containing 0.5% Triton X-100) at room temperature (phalloidin). Subsequently, cells were washed three times with PBS-T and blocked with 0.5% bovine serum albumin in PBS for 30 min at room temperature. Cells were incubated for 1 h at 37 °C with 1:50 anti-Src 327, 1:50 anti-Fak (A17), 1:100 anti-paxillin, and 1:50 phalloidin. Coverslips were washed twice for 5 min with PBS-T, and then incubated with the corresponding secondary antibody for 1 h at 37 °C, washed again, and mounted in Mowiol medium. Cells were examined using an Axiosvert 200 inverted microscope (Zeiss, Göttingen, Germany) equipped with a color digital camera (Spot RT Slider) (Diagnostic Instruments, Sterling Heights, MI) with a 63/1.40 oil Plan-Apochromat objective. Confocal analysis was performed with a Leica TCS SP2I Spectroscopic microscope and 63X/1.3 NA oil objective.

**Immunocytochemistry**—Exponentially growing cells in complete medium in the presence or absence of Doxy during 48 h detached with trypsin, washed twice with complete medium to neutralize the protease, then washed twice with PBS to remove serum, and the final pellet of cells was fixed in formol. The fixed pellet was included in paraffin and 5-µm sections were prepared. The paraffin sections were treated accordingly (29) for immunostaining with mAb 327 to detect total c-Src or with mAb EC10 to detect chicken c-SrcDN.

**Evaluation of the Anti-tumorigenic Activity of the Src Double Mutant**—Female athymic nude-Foxn1 null mice 6–8 weeks old, purchased from Harlan (Indianapolis, IN), were used. Mice were housed under pathogen-free conditions in a temperature-controlled room on 12/12 h light/dark schedule with food and water ad libitum. All procedures involving animals and their care were in accordance with national and international regulations. Each nude mice was implanted each with a 1.7 mg of 17β-estradiol pellet (90 days release, Innovative Research of America) to supplement the estrogens requirement for MCF-7 proliferation. A week after implantation of 17β-estradiol pellet, 5 × 10^6 viable cells were subcutaneously injected in both back flanks to obtain solid tumors. Mice were divided into three experimental groups of 8–9 animals. Group 1 (control) never received Doxy with the drinking water, group 2 received Doxy with the drinking water (2 mg/ml) from the first day after injection of MCF-7 Tet-on SrcDN cells, and group 3 received Doxy with the drinking water (2 mg/ml) 6 weeks after the injection of tumor cells. The bottles of water were changed every 3 days. The tumors were measured by vernier caliper, weekly, and their volumes were calculated using the formula LxWxHx0.52.

**Immunohistochemistry of Xenografts**—Tumors from untreated and Doxy-treated mice were removed after cervical dislocation twelve weeks after tumor cell implantation. Tumor specimens
were frozen in liquid nitrogen and/or fixed in 4% formaldehyde solution immediately after surgical resection. Fixed samples were paraffin embedded (29), and 5-μm sections were analyzed by hematoxylin/eosin staining, as described (29). Immunohistochemical staining for Ki67, c-Src, and avian c-Src was also performed. The number of Ki67 and total c-Src positive cells was estimated in six high-power fields containing 60–80 cells/field (×200). Results were classified as follows: 4 (≥75%); 3 (50–75%); 2 (25–50%); 1 (≤25%); and 0 (no or weak staining in a few cells). Frozen samples were processed for Western blot analysis of PARP, total c-Src and avian Src expression.

**Statistical Analyses**—Analyses were performed with the SPSS/PC version 12.0X statistical package (SPSS, 2005, Chicago, IL). Comparisons between groups were performed by the Mann-Whitney U, Wilcoxon, Anova and chi2 tests. When the expected frequency of any group was lower than 5, the Fisher exact test was used. All the tests applied were two-tailed and \( p < 0.05 \) was considered significant.

**RESULTS**

**Inducible Doxycycline Expression of SrcDN in MCF7 Cells** Results in Phenotypic Changes—Clone 89rtTA of MCF7 cells expressing the reverse tetracycline transactivator (rtTA) maintained in the presence of G418 (0.2 mg/ml) (26) was cotransfected with pTET-Splice-SrcDN and pBABE-puromycin. Cells maintained in complete medium were selected in the presence of G418 (0.2 mg/ml) with 2 μg/ml of puromycin. Resistant clones were expanded and tested by Western blotting for inducible expression of avian c-Src dominant negative mutant (SrcDN) upon addition of Doxy (0.2 and 2 μg/ml) to cultures for 48 h. Using the mAb EC10, which only recognizes the avian c-Src mutant, but not the endogenous human c-Src expressed by MCF7 cells, four MCF7-Tet-On-SrcDN clones were selected for the following studies (Fig. 1A). Total Src expression was also analyzed by Western blot using mAb 327 (Fig. 1A). Cells maintained in complete medium were treated with increasing concentrations of Doxy for 48 h to determine the dose-dependent induction of SrcDN. The concentration of Doxy chosen to perform the following assays was 2 μg/ml as the results showed an appropriate induction of SrcDN in all selected clones; higher concentrations of Doxy did not increase SrcDN expression (data not shown). We also determined the kinetics of SrcDN expression by collecting cells after incubation with Doxy (2 μg/ml) for different periods of time, and observed maximal SrcDN expression between 48 and 72 h (data not shown). Next, we analyzed the expression of SrcDN in cells by immunocytochemistry (see “Experimental Procedures”). Consistent with results obtained by Western blot (Fig. 1A), expression of SrcDN was induced only by the addition of Doxy (Fig. 1B, SrcDN EC10). In the presence of Doxy, staining of Total Src increased as a consequence of additional expression of SrcDN (Fig. 1B, Total-Src (327)). It is worth mentioning that
Src and Breast Cancer Tumorigenesis

SrcDN was mainly found at the plasma membrane where it is biologically functional (Fig. 1B).

We observed that SrcDN expression caused cells to become refractive and rounded as compared with cells grown in the absence of Doxy (Fig. 1C, and control cells (MCF7-Tet-On: Clone 89rtTA of MCF7 cotransfected with empty pTET-Splice and pBABE-puromycin; supplemental Fig. S1A). These results suggest that expression of SrcDN interferes with cell adhesion and with the correct assembly and dynamics of the cytoskeleton. The following experiments were carried out with all four clones obtaining similar results. The data presented here are referred to clone 2.

SrcDN Induces Relocation of Fak, Paxillin, and c-Src—As described above, SrcDN expression caused morphological changes as well as inhibition of migration, adhesion and spreading. Therefore, we next analyzed if it also altered the cellular distribution of focal adhesion molecules involved in the control of such processes (4, 32). Fluorescence microscopy analysis showed that c-Src, Fak, and paxillin in the absence of Doxy were mainly located at focal contacts homogeneously distributed along the membrane of spread cells. Phalloidin staining revealed the normal assembly of cytoskeleton actin stress fibers (Fig. 3, −Doxy). Interestingly, expression of SrcDN by addition of Doxy to cell cultures caused drastic changes in the distribution of these proteins (Fig. 3, +Doxy). c-Src appeared evenly expressed at the plasma membrane of the cell, and the normal actin fiber assembly was disrupted. Fak and paxillin accumulated in patches, indicating large peripheral adhesions. The pattern of distribution of these proteins was unaltered when control cells were treated with Doxy (supplemental Fig. S2).

To further analyze the proteins involved in the assembly of large focal adhesions found in cells expressing SrcDN, colocalization analyses of c-Src, Fak, and paxillin staining were performed with control cells incubated in the presence or absence of Doxy for another 24 h. MCF7 cells expressing SrcDN (Fig. 2A, +Doxy) clearly showed a reduced capacity to migrate into the wounded area when compared with cells in which the mutant was not induced (Fig. 2A, −Doxy). The wound repair assay was also performed with control cells incubated in the presence or absence of Doxy but no differences in migration were observed (supplementary Fig. S1B).

Tumor cell adhesion to extracellular matrix components such as fibronectin and the basement membrane has been shown to be an important step in cell migration and cell survival (30, 31). We next examined whether the expression of SrcDN altered cell adhesion to fibronectin coated wells, and cell spreading (see “Experimental Procedures”). The expression of SrcDN caused a significant reduction in cell adhesion to fibronectin (42%, p < 0.01) as compared with Doxy-untreated cells (Fig. 2B). Similar results were obtained for direct adhesion to plastic (data not shown). Additionally, the expression of SrcDN inhibited cell spreading (Fig. 2C; 94.06%, p < 0.01). Cell adhesion and spreading was not affected when the control cells were incubated in presence of Doxy (supplementary Fig. S1, C and D).

FIGURE 2. Effect of SrcDN on cell migration, adhesion and spreading. A, cell migration was determined by wound repair assays. MCF7-Tet-On-SrcDN cells were incubated in the absence or presence of Doxy (2 μg/ml) for 24 h, then the wound was generated (0 h), and cells were incubated for 24 h with or without Doxy (24 h), and cell migration into the wound was analyzed by phase-contrast microscopy (magnification × 200). To study cell adhesion and spreading, MCF7-Tet-On-SrcDN cells were pretreated with or without Doxy (2 μg/ml) for 48 h and seeded in 96-well plates precoated with fibronectin (1 mg/ml). After 2 h of incubation at 37 °C, the percentage of adherent (B) or spread (C) cells was determined as described under “Experimental Procedures.” Four independent experiments were carried out in triplicate. **, p < 0.01 as compared with the control (ANOVA test).
However, it did not alter the colocalization of these proteins at focal contacts, which concentrated into large patches at the cell surface (Fig. 4).

**SrcDN Impairs Activation and Downstream Effects of the Src-Fak Complex**—The c-Src-Fak complex controls different signaling pathways triggered by growth factors or by integrins (32), which in turn regulate proliferation, survival, migration, and cell adhesion. Because expression of c-Src mutant impaired cell migration, adhesion, and spreading, we further analyzed whether it also affected the activation of the Src-Fak complex and its downstream substrates.

Growth factor or integrin stimulation induces Fak autophosphorylation on Tyr925, generating a high affinity binding site for Src SH2 domain (33). Thus, we then determined the extent of Fak-Tyr925 phosphorylation in exponentially growing cells in the presence or absence of Doxy (2 μg/ml) for 48 h. Interestingly we observed that expression of SrcDN increased the degree of Fak-Tyr925 phosphorylation (Fig. 5A). A possible explanation for this result could be that the overexpressed SrcDN competes and displaces endogenous c-Src from its interaction with P-Tyr925-Fak, thus, protecting it from dephosphorylation. Consistently, the amount of total Src bound to Fak-Tyr925 increased in cells treated with Doxy (Fig. 5D, IP Fak/WB c-Src), suggesting that the observed increased phosphorylation of Fak-Tyr925 in Doxy-treated cells could be caused by SrcDN SH2 domain protection from dephosphorylation. Alternatively, the binding of SrcDN may induce a greater percentage of Fak molecules to autophosphorylate, thereby increasing the Fak-Tyr925 signal.

Once the association of Src with Fak has occurred, Src suffers a conformational change leading to Src activation and further phosphorylation of Fak on Tyr residues, which is required for full activation of Fak. Among these residues is Tyr925, which is exclusively phosphorylated by Src (4). To determine if Fak was activated in SrcDN overexpressing cells, the phosphorylation of Fak on Tyr925 was assessed. As expected, Fak-Tyr925 phosphorylation was diminished in Doxy-treated cells (Fig. 5A).

Because the activated Fak-Src complex recruits and phosphorylates proteins involved in cell adhesion, actin dynamics, proliferation, and survival, we determined the extent of paxillin and p130CAS phosphorylation in exponentially growing cells in the absence or presence of Doxy as before. pY-paxillin and pY-p130CAS were clearly diminished upon addition of Doxy to cultures (Fig. 5, B and C). Likewise, the amount of p130CAS associated with Fak was drastically decreased (Fig. 5D, IP Fak/WB p130CAS).
Activation of the Src/Fak complex also causes association and activation of PI3K (34, 35). We then analyzed whether SrcDN expression altered the association of p85-PI3K with Fak. To this end, Fak was immunoprecipitated from exponentially growing cells in the absence or presence of Doxy (2 μg/ml) for 48 h, and the presence of p85-PI3K in Fak immune complex was determined by Western blot. The results showed that in the absence of Doxy p85-PI3K was associated with Fak (Fig. 5D, IP Fak/WB p85). However, expression of SrcDN abolished this interaction (Fig. 5D, IP Fak/WB p85).

Together, these results indicate that overexpression of SrcDN seems to be enough to inactivate the Src-Fak complex, which in turn is involved in the control of cell migration, adhesion, and spreading of breast cancer cells.

Reduction of Cell Proliferation Rate by SrcDN—c-Src expression and function has been reported as an important mediator of proliferation for a variety of cancer cells (1, 36); however, it is not involved in colon cancer cells proliferation (4), albeit c-Src overexpression in these tumors (11). We have analyzed whether overexpression of the dominant negative form of c-Src in MCF7 breast cancer cells could alter serum- and EGF-stimulated proliferation in vitro. For this purpose serum-starved and exponentially growing cells in serum or EGF-supplemented medium were incubated in the absence or presence of Doxy (2 μg/ml) for 72 h. Afterward, cell proliferation was assessed (Fig. 6A). The induction of SrcDN expression significantly reduced the proliferation rate of serum-starved and exponentially growing cells in serum as well as in the EGF supplemented medium (Fig. 6A, p < 0.05). No significant differences in proliferation rate were found when control cells were incubated in the presence or absence of Doxy (supplemental Fig. S1).

To define the effects of SrcDN expression on cell cycle progression, BrdU pulse-label experiments were performed in untreated and Doxy-treated cells. In the absence of Doxy, cells progressed through the cycle, and after 36 h, the BrdU-labeled cells showed tendency toward homogeneous distribution in G1, S, and G2/M phases (Fig. 6B). In contrast, SrcDN expression caused cell accumulation in the G1 phase and decreased BrdU-labeled cells in S and G2/M (Fig. 6B). The ratio between the percentages of untreated and Doxy-treated cells in each phase at each time was calculated (Fig. 6B, ratio ± Doxy) and clearly showed that SrcDN caused cells to accumulate in the G1 phase, whereas cells in S and G2/M diminished with time (Fig. 6B, ratio ± Doxy). These results indicate that c-Src is involved in the control of G1/S transition in exponentially growing breast cancer cells.

SrcDN Reduces Serun and EGF-mediated Activation of Erk1/2 and Akt, and Up-regulates p27kip1 Expression—Once we demonstrated the relevance of active c-Src for breast cancer...
Src and Breast Cancer Tumorigenesis

A

|  | Doxy |  |  |
|---|---|---|---|
| pY397-Fak |  | +  | + |
| pY925-Fak |  | +  | + |
| Fak |  |  |  |

B

|  | Doxy |  |  |
|---|---|---|---|
| pY-Paxillin |  | +  | + |
| Paxillin |  |  |  |

C

|  | p130CAS, and p85-P13K |  |  |
|---|---|---|---|
| IP p130CAS/WB pY |  |  |  |
| Fak |  |  |  |

D

|  | p130CAS, and p85-P13K |  |  |
|---|---|---|---|
| IP p130CAS/WB pY |  |  |  |
| Fak |  |  |  |

FIGURE 5. Effects of SrcDN on the activation and interaction of Fak, paxilllin, p130CAS, and p85-P13K. A, the effect of SrcDN expression on Fak-Tyr397 and Fak-Tyr925 phosphorylation was determined by Western blot with total cell extracts of exponentially growing cells in complete medium in the absence or presence of Doxy (2 μg/ml) for 48 h. B, the effect of SrcDN expression on paxillin phosphorylation was analyzed in cell lysates from cultures (as described in A) immunoprecipitated (IP) with an anti-paxillin antibody and immunoblotted with an anti-phosphotyrosine mAb 4G10. C, the same experiment was carried out to study p130CAS phosphorylation. D, Fak interactions were analyzed by performing Fak IP and Western blot detection of Src, p130CAS, and p85-P13K determined by the immune complexes, as described under “Experimental Procedures.”

cell proliferation in vitro, we analyzed its role in signaling pathways leading to cell proliferation and survival. Serum-starved cells were incubated in the absence or presence of Doxy (2 μg/ml) for 48 h. Cells were stimulated with serum (10%) or with EGF (100 ng/ml) for different time periods, and activation of Erk1/2 and Akt and p27Kip1 expression were determined by Western blot. Both serum and EGF activated Erk1/2 reaching maximal phosphorylation after 15 min and declining toward basal levels after 1 h (Fig. 6C). Expression of SrcDN caused a slight reduction of serum and EGF-mediated Erk1/2 activation at 15 min but produced a marked decrease at later times (Fig. 6C), suggesting that SrcDN exerts its greatest effect at late stages of mitogen-activated protein kinase signaling. Additionally, stimulation of Akt by serum or by EGF was drastically diminished in Doxy-treated cells (Fig. 6C). This result is consistent with the reduced association of p85-P13K to the Fak-Src complex previously observed (Fig. 5D). In agreement with the reduced rate of cell proliferation observed upon expression of SrcDN (Fig. 6A), the expression of cyclin-Cdk inhibitor p27kip1 was highly increased in both serum and EGF-stimulated cells (Fig. 6C).

Reversibility of the Effects of Inducible Expression of SrcDN in MCF7 Cells—To test whether the effects of SrcDN expression could be reverted, we first analyzed the expression of total Src and SrcDN by Western blot in exponentially growing cultures of MCF7-Tet-On-SrcDN in complete medium in the absence or presence of Doxy (2 μg/ml) for 48 h versus 24 h depletion of Doxy from the 48 h Doxy-induced cells. As observed in Fig. 7A, SrcDN induction was observed as before (Fig. 1A) after 48 h of Doxy induction. However, when cultures were washed with prewarmed culture medium and then incubated 24 h in the absence of Doxy SrcDN expression was abrogated (Fig. 7A). Consistent with the reversible expression of SrcDN, the refractive and rounded phenotype of cells upon induction of SrcDN recovered their normal epithelial phenotype when SrcDN was no longer expressed (Fig. 7B). Moreover, the signaling events triggered by SrcDN expression also reverted 24 h after the removal of Doxy. Thus, Doxy depletion reduced Fak phosphorylation at Tyr397 and increased Tyr925 phosphorylation to levels of non-induced cells (Fig. 7C).

Effect of c-Src-siRNA in MCF7 Cell Migration, Attachment, Spreading, and Proliferation—MCF-7 cells constitutively expressing c-Src-siRNA were generated to complement the studies performed with the SrcDN inducible mutant. EGFP-siRNA was used as control because it does not affect biological responses of eukaryotic cells (27, 37). First, we analyzed the effect of c-Src-siRNA on endogenous c-Src expression. As compared with control cells expressing EGFP-siRNA, c-Src-siRNA expression in MCF7 cells strongly reduced the endogenous c-Src levels (Fig. 8A). Then we analyzed the biological effects of c-Src-siRNA expression in serum-stimulated versus EGFP-siRNA expressing cells. MCF7 cells expressing c-Src-siRNA showed a significant reduction of migration, attachment, spreading, and proliferation as compared with control cells (Fig. 8, B–E, p < 0.05), supporting the important role of c-Src in these biological responses.

Role of c-Src in Tumorigenesis—Because the anti-proliferative action of SrcDN mutant in vitro has been clearly established (Fig. 6), we next examined its inhibitory action in vivo. For this purpose, we analyzed whether overexpression of SrcDN under Doxy induction could alter the tumorigenic potential of MCF7 carcinoma cells when inoculated in female athymic nude-Foxn1nu mice. Because MCF7 cells are estrogen dependent for growth in vivo, mice were implanted with a 17β-estradiol pellet (90 days release) 1 week before cell injection. Animals were separated into three different groups: group 1 (never received Doxy in the drinking water; control group), group 2 (receiving 2 mg/ml of Doxy in the drinking water from the first day after implantation of cells) and group 3 (maintained without Doxy for the first 6 weeks, and then treated with 2 mg/ml of Doxy in the drinking water for the following 6 weeks). The experiment was carried out for 12 weeks (the period of time that estrogen pellets are active), and tumor size was measured weekly. At the end, tumors were excised and processed for biochemical and histological analyses.

Although there was not a time delay in the appearance of tumors between groups 1 and 2, the number of tumors developed in the group of mice receiving Doxy (group 2) was significantly smaller than in group 1 (control group, Fig. 9, A and B, *, p < 0.05). We next analyzed whether SrcDN expression could cause regression of tumors once they were established. To this end, tumors from groups 1 and 3 were compared after 6 and 12 weeks. The number of tumors within group 1 at 12 weeks was slightly increased when compared with the values at 6 weeks, although there were not significant differences (Fig. 9, A and B).
However, there was a significant reduction in tumor number when comparing values at 12 versus 6 weeks in group 3 (Fig. 9, A and B, #, \( p < 0.05 \)). Consistent with the inhibition of tumorigenesis and tumor regression, the c-Src expression in tumors was determined by immunohistochemical analysis (Fig. 9C), and the quantifications showed a significant increase in groups 2 and 3 because of the SrcDN expression when compared with group 1 (Fig. 9B, †, \( p < 0.05 \)). The immunohistochemical analysis of Src revealed the expected pattern of staining, which was restricted to the plasma membrane of inoculated breast cancer cells (Fig. 9C). This distribution of c-Src was consistent with immunocytochemistry of in vitro growing cells in presence or absence of Doxy (Fig. 1B). The inhibition of tumorigenesis was associated with a significant decrease in the proliferation of tumors belonging to groups 2 and 3 versus group 1, as revealed by immunohistochemical detection of Ki67 in tumors (Fig. 9, B, †, \( p < 0.05 \), and C). Concomitantly, apoptosis was significantly increased in SrcDN-expressing tumors (groups 2 and 3), as determined by cleavage of native poly (ADP-ribose) polymerase (PARP) p116 into PARP p85 in tumor biopsies by Western blot (Fig. 9D). The p116/p85 ratio calculated from the Western blot showed a significant decrease in the tumors of mice treated with Doxy (Fig. 9B, *, \( p < 0.05 \)), indicating a higher degree of apoptosis upon expression of SrcDN. p53 levels were also determined by Western blot in tumors extracts and found clearly increased in mice belonging to groups 2 and 3 as compared with group 1. Given that p130CAS cleavage has been associated with anoikis and suppression of tumor growth (38), to further elucidate the apoptosis mechanism, p130CAS degradation was determined by Western blot in tumor extracts. In groups 2 and 3, a clear increment of a lower molecular mass protein (~83 kDa) immunoreactive to the p130CAS antibody was observed when compared with
Src and Breast Cancer Tumorigenesis

A

- Doxy  + Doxy  +/- Doxy
SrcDN
Total-Src
α-tubulin
EC10 mAb
327 mAb

B

- Doxy  + Doxy  +/- Doxy
MCF7-Tet-On-SrcDN

C

- Doxy  + Doxy  +/- Doxy
pY397-Fak
pY925-Fak
Fak

FIGURE 7. SrcDN-mediated effects are reverted by down-regulation of SrcDN expression in MCF7 cells. A, expression of avian SrcDN (anti-c-Src mAb EC10) and total Src (327 mAb) was determined by Western blot with 25 μg of protein lysates from exponentially growing MCF7-Tet-On-SrcDN cells in the absence (-Doxy) or presence of 2 μg/ml Doxy (+Doxy) for 48 h versus 48 h Doxy-induced cells subsequently depleted of Doxy for 24 h (+Doxy). Membranes were reblotted with anti-α-tubulin for loading control. B, morphological analysis of cultures treated as above by phase contrast microscopy (magnification ×200). C, Fak phosphorylation at Tyr397 and Tyr925 was determined by Western blot with 25 μg of protein lysates from the cultures described above. Membranes were reblotted with anti-Fak for loading control.

Together these results showed that expression of SrcDN is able to reduce tumor development and to induce tumor regression once they were established. These effects are associated with increased apoptosis in tumors expressing SrcDN and with a reduction in cell proliferation.

DISCUSSION

Because the inherited or acquired deregulation of protein kinase activity has been implicated in the pathogenesis of many human diseases, including cancer, the inhibition of these enzymes has been postulated as a promising strategy for anti-cancer treatment, among them is the non-receptor tyrosine kinase c-Src (36, 41, 42).

c-Src controls pathways involved in cellular functions as diverse as cell growth, migration, adhesion, and survival (1) and has been found to be overexpressed or constitutively active in a large percentage of tumors, including breast and colon cancers unable to phosphorylate Fak or Src-Fak complex substrates.

The induction of SrcDN in MCF7 led to morphological changes suggesting an aberrant function of focal adhesion assembly and cytoskeleton dynamics. When we further analyzed the effects of SrcDN expression on cellular functions that depend on the integrin signaling pathways and cytoskeleton machinery, such as cell migration, adhesion and spreading, we found them clearly impaired upon induction of the mutant. These alterations were also observed in MCF7 cells when c-Src was knocked-down by siRNA.

The morphological changes induced by SrcDN expression were associated with relocation of c-Src, Fak, and paxillin as well as with disruption of normal actin fiber assembly. SrcDN expression did not alter c-Src, Fak and paxillin co-localization but caused their rearrangement into large focal adhesions. This assembly of large, peripherally localized adhesions has been previously observed in fibroblasts. Fibroblasts from c-Src−/− mice show decreased rate of migration and spreading, and increased size of peripherally localized adhesions (45). Also, the expression of Src kinase defective mutants in normal fibroblasts results in large peripheral adhesions (46). It has been previously suggested that increased number and size of adhesions in Fak−/− and Src−/− cells could be caused by an inhibition of adhesion turnover (45).
An important role of the Fak/Src complex in the regulation of the rate of focal adhesion assembly and disassembly has been postulated. In fact, a recent study in fibroblasts has shown that Fak, Src, and their downstream substrates p130Cas, paxillin, Erk1/2, and MLCK are required for adhesion disassembly (32). When we analyzed if the aberrant assembly of focal adhesions in SrcDN expressing MCF7 cells was linked to effects on activation of Src-Fak complex and its downstream substrates, we found increased Fak autophosphorylation on Tyr397 associated with augmented co-immunoprecipitation of Src. Increased phosphorylation of Fak on Tyr397 has also been recently observed in KM12C colon carcinoma cells expressing Src inactive mutants (4). In agreement with these authors, we suggest that the expressed SrcDN, via its SH2 domain, may stabilize autophosphorylated Fak protecting it from dephosphorylation. On the other hand, when we analyzed Fak phosphorylation on residue Tyr925, which is exclusively catalyzed by Src and required for adhesion turnover (4), we found it diminished in SrcDN-expressing cells. Also, phosphorylation of p130Cas and paxillin, essential for adhesion assembly/disassembly and actin dynamics (32), was diminished in SrcDN-expressing MCF7 cells. From these results, we conclude that expression of the SrcDN mutant, which is able to interact with P-Tyr397-Fak but is catalytically inactive, alters the Fak/Src complex control of cell adhesion, spreading and migration. The inactivation of the Fak/Src complex in SrcDN-expressing cells could be explained by SrcDN kidnapping of autophosphorylated Fak and subsequent inability to further phosphorylate Fak and Src-Fak substrates.

Expression of SrcDN was able not only to impair oncogenic properties of breast cancer cells such as migration, adhesion, and spreading resembling the effects of this
mutant on colon cancer cells (4), but it was also efficient to reduce cell proliferation in vitro and in vivo, which was not observed in colon cancer cells (4). Our data show that induction of SrcDN expression significantly reduced the proliferation rate of serum-starved and exponentially growing MCF-7 cells in serum and in EGF-supplemented medium, causing accumulation of cells in G1 phase of the cell cycle. The inhibition of proliferation was associated with reduced activation of Akt after serum or EGF treatment and a marked increase in p27kip1 expression. These data are in agreement with our previously reported results on W53 lymphoid cells cultivated in the presence of Src inhibitor PP1 (8). The relevance of c-Src in proliferation of breast cancer cells was confirmed by depletion of this oncoprotein by means of siRNA expression.

The inhibition of Akt activation by SrcDN described here, is consistent with Akt inhibition and the reduction of cell proliferation. These results are in agreement with those obtained in TGF-β1 treated hepatoma cells, where inhibition of c-Src by PP2 augmented levels of p27kip1 (53). Also, in ErbB2-overexpressing breast cancer cells, Herceptin causes increased expression of p27kip1 by inhibiting PL3K and Akt (54). These effects can be linked to the inhibition of association and activation of c-Src with ErbB2 (22).

Proliferation of cancer cells in vitro does not always correlate with their tumorigenic potential. We therefore tested the anti-tumorigenic capacity of SrcDN in MCF7 cells inoculated in estrogen-primed nude mice. Our results showed that SrcDN expression significantly diminished the number of tumors and, even more, induced their regression once they were established. Interestingly, whereas treatment of cells growing in vitro for

FIGURE 9. Effect of conditional expression of SrcDN in tumorigenesis and tumor regression. A, the number of tumors per mouse in each group was measured weekly. Inhibition of tumorigenesis was evaluated by comparison between groups 1 and 2 and tumor regression by comparing groups 1 and 3 (data quantification is shown in B). Representative immunohistochemistry analysis of tumors from each group detecting total c-Src (mAb 327) and Ki67 expression at the end of the experiment and quantified (see B) as described under “Experimental Procedures.” D, PARP and p130CAS cleavage, and p53 and VEGF expression analyzed by Western blot in homogenized tumor tissue. PARP p116 and p85, and p130CAS p130 and p83 expression were quantified by densitometry and expressed as PARP p116/p85 and p130CAS p130/p83 ratios; p53 and VEGF protein was quantified and normalized with tubulin as loading control (data are shown in B). Statistical significances were calculated as described under “Experimental Procedures.” #, p < 0.05 (Wilcoxon); †, p < 0.05 (U-Mann Whitney); and ††, p < 0.05 (Pearson chi²).
48–72 h with Doxy caused a clear reduction of proliferation rate, apoptosis was not observed, although inhibition of the PI3K-Akt signaling pathway may precede apoptotic events that would take place later on. In contrast, the induction of SrcDN in growing breast cancer cells in vivo led not only to reduction of their proliferation, but also to increased apoptosis, as shown by increased p53 expression and PARP and p130CAS degradation. p130CAS cleavage has been recently related to anoikis and suppression of tumor growth upon induction of Fak-Y397F in Fak−/− fibroblasts transformed by v-Src (38). The involvement of p130CAS cleavage in anoikis is also supported by other studies (55–60).

In addition with PARP and p130CAS cleavage, and increased p53, we also observed decreased VEGF levels in SrcDN-expressing tumors. These effects are associated with augmented apoptosis and diminished tumorigenesis. Consistently with these results it has been previously shown, in other human tumor cells, that v-Src and p53 exert opposed effects on VEGF expression, whereas v-Src increases VEGF, p53 decreases it (61).

In conclusion, the data presented in this manuscript highlight the relevance of c-Src in Src-Fak complex control, which in turn regulates essential cellular processes, like migration, adhesion, spreading, and cell growth. In fact, expression of a c-Src mutant (SrcDN), which favors the interaction with Fak but has no kinase activity, is enough to impair cellular functions required for breast cancer growth and even to cause significant tumor regression. Additionally, we have studied the role of c-Src by silencing its expression with siRNA and obtaining similar effects to those induced by SrcDN expression. These data demonstrate the key involvement of c-Src in the biology of breast cancer cells, suggesting that tumors may become addicted to Src function. Thus, we consider that c-Src is a potential target for breast cancer therapy.

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