Integrin $\beta 3$ -mediated Src activation regulates apoptosis in IEC-6 cells via Akt and STAT3

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Intestinal epithelial (IEC-6) cells are resistant to apoptosis following the inhibition of ODC (ornithine decarboxylase) and subsequent polyamine depletion. The depletion of polyamines rapidly activates NF- κ B (nuclear factor κ B) and STAT3 (signal transducer and activator of transcription 3), which is responsible for the observed decrease in apoptosis. Since both NF- κ B and STAT3 signalling pathways can be activated by Src kinase, we examined its role in the antiapoptotic response. Inhibition of ODC by DFMO (α -difluoromethylornithine) increased the activity of Src and ERK1/2 (extracellular-signal-regulated kinase 1/2) within 30 min, which was prevented by exogenous polyamines added to the DFMO-containing medium. Conversely, epidermal growth factor-mediated Src and ERK1/2 activation was not prevented by the addition of polyamines. Inhibition of Src with PP2 {4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine} and a DN-Src (dominant-negative Src) construct prevented the activation of Akt, JAK (Janus kinase) and STAT3. Spontaneous apoptosis was increased in DN-Src-expressing

cells and the protective effect of polyamine depletion was lost. Polyamine depletion by DFMO increased integrin $\beta 3$ Tyr⁷⁸⁵ phosphorylation. Cells plated on fibronectin had significantly higher $\beta 3$ phosphorylation and Src activation compared with plastic. Exogenous polyamines added to the fibronectin matrix prevented Src activation. Arg-Gly-Asp-Ser inhibited $\beta 3$, Src and Akt phosphorylation and sensitized polyamine-depleted cells to tumour necrosis factor α /cycloheximide-mediated apoptosis. Fibronectin activated Src and subsequently protected cells from apoptosis. Together, these results suggest that the inhibition of ODC rapidly removes a small pool of available polyamines triggering the activation of $\beta 3$ integrin, which in turn activates Src. The subsequent Akt and JAK activation is accompanied by translocation of NF- κ B and STAT3 to the nucleus and the synthesis of antiapoptotic proteins.

Key words: $\beta 3$ integrin, caspase 3, α -difluoromethylornithine (DFMO), intestinal epithelial cell, polyamine, Src.

INTRODUCTION

Apoptosis is a genetically determined process of programmed cell death essential to controlling cell number in all replicating tissues, including those of developing embryos as well as those of adults. Our laboratory has been involved for some time in examining apoptosis in intestinal epithelial cells. We have studied this process in both mice and in a line of cultured intestinal epithelial cells (IEC-6), a non-transformed line derived from adult rat crypt cells [1]. The lining of the small intestine is one of the most rapidly turning over tissues in the body. Apoptosis is responsible for eliminating extra stem cells and, therefore, controlling cell number in the intestine [2,3]. We have shown that the depletion of polyamines inhibits γ -radiation-induced apoptosis in intestinal epithelial cells of mice and in IEC-6 cells induced by radiation, camptothecin or TNF α (tumour necrosis factor α)/CHX (cycloheximide) [4–6]. Caspase activation and apoptosis occur via two major pathways. An extrinsic pathway, stimulated by TNF α , a pleiotropic cytokine, activated upon binding of ligand recruits and activates procaspase 8 [7]. In IEC-6 cells, polyamine depletion inhibits the activation of caspase 8 [6]. The second major pathway is the intrinsic or mitochondrial pathway, which results in the release of cytochrome *c* from mitochondria and the subsequent formation of an apoptosome with Apaf (apoptotic protease-activating factor) and procaspase

9 [7]. This results in the formation of active caspase 9, which in turn activates caspase 3. We have shown that polyamine depletion inhibits cytochrome *c* release, caspase 9 activation and its subsequent activation of caspase 3 [8]. Another group of proteins known as IAPs (inhibitors of apoptosis proteins) prevent apoptosis by directly inhibiting caspases [9]. The intracellular levels of the Bcl-2 (B-cell lymphocytic-leukaemia proto-oncogene 2) family proteins and IAPs are in large part determined by the activity of the antiapoptotic transcription factors, NF- κ B (nuclear factor κ B) and STAT3 (signal transducer and activator of transcription 3) [10,11].

The polyamines, spermidine and spermine, and their precursor, putrescine, are found in virtually all cells of higher eukaryotes [12] and are intimately involved in, and required for, cell growth and proliferation [13,14]. Intracellular polyamine levels are highly regulated and depend primarily on the activity of ODC (ornithine decarboxylase), which catalyses the first rate-limiting step in polyamine biosynthesis, the decarboxylation of ornithine to form the diamine putrescine [15]. The second rate-limiting enzyme, *S*-adenosylmethionine decarboxylase, forms *S*-adenosyl homocysteine, which provides the propylamine groups to synthesize spermidine from putrescine and spermine from spermidine. We have used DFMO (α -difluoromethylornithine) to inhibit ODC and deplete cells of polyamines. DFMO is a specific and irreversible inhibitor of ODC, having no effects except those caused

Abbreviations used: Bcl-2, B-cell lymphocytic-leukaemia proto-oncogene 2; CHX, cycloheximide; cIAP2, cellular inhibitor of apoptosis protein 2; FBS, fetal bovine serum; dFBS, dialysed FBS; DFMO, α -difluoromethylornithine; DMEM, Dulbecco's modified Eagle's medium; DN-Src, dominant-negative Src; DPBS, Dulbecco's PBS; ECL, enhanced chemiluminescence; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; IAP, inhibitor of apoptosis protein; JAK, Janus kinase; NF- κ B, nuclear factor κ B; ODC, ornithine decarboxylase; PARP, poly (ADP-ribose) polymerase; PI3K, phosphoinositide 3-kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; RGD, Arg-Gly-Asp; RGDS, Arg-Gly-Asp-Ser; SH3 domain, Src homology 3 domain; STAT3, signal transducer and activator of transcription 3; TBS, Tris-buffered saline; TNF α , tumour necrosis factor α .

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by the inhibition of ODC and the subsequent decrease in intracellular polyamines [15,16]. This has been demonstrated in many experiments in which the inhibitory effects of DFMO on cell proliferation, migration and apoptosis have been prevented by the addition of exogenous polyamines [16–18].

It is unlikely that a decrease in polyamines activates these two signalling pathways independently of each other. Many surface receptors contain relatively short cytoplasmic domains and lack intrinsic catalytic activity. These domains possess binding sites for non-receptor tyrosine kinases such as Src [19]. Src kinases are activated by integrins, growth factor stimulation and a variety of G-protein-coupled receptors. Activation of Src prevents apoptosis and is a contributing factor to the development of many cancers. In hepatocytes, survival signals induced by TGF- β 1 (transforming growth factor β 1) are mediated by the Src induction of the Akt pathway [20]. Progesterone induced Src activation of STAT3 via a JAK (Janus kinase)-dependent mechanism in breast cancer cells, and expression of a dominant-negative STAT3 inhibited tumour formation [21]. Activation of Src has been shown to prevent anoikis of intestinal epithelial cells by increasing the expression of Bcl-X_L [22].

Recent studies have demonstrated that integrin β cytoplasmic domains regulate integrin signalling through Src-family kinases [23]. Src-family kinases are activated downstream of integrins as well as downstream of other receptors, and they are required for integrin signalling [24]. c-Src was found to directly interact with the β 3 integrin cytoplasmic domain and was involved in the initiation of outside-in signalling [23,24]. Given the fact that Src kinases are involved in regulating multiple downstream targets of integrin [24], including FAK (focal adhesion kinase), PI3K (phosphoinositide 3-kinase)/Akt and MAPK (mitogen-activated protein kinase), the role of polyamines in integrin-mediated Src activation could serve as a paradigm for polyamine-mediated signal transduction pathways. We, therefore, sought to understand the mechanism by which Src is activated following polyamine depletion. We have shown here that the inhibition of ODC by DFMO rapidly activates β 3 integrin by phosphorylating it at Tyr⁷⁸⁵, which in turn activates Src in IEC-6 cells, and that this activation precedes that of Akt and STAT3. Furthermore, inhibition of Src either by PP2 {4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine}, DN-Src (dominant-negative Src) or integrin antagonists prevents the activation of these pathways and increases apoptosis in response to TNF α /CHX.

EXPERIMENTAL

Reagents, constructs and antibodies

Cell culture wares were purchased from Corning Glass Works (Corning, NY, U.S.A.). Media and other cell-culture reagents were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Fibronectin-coated cellware was obtained from BD Biosciences (Bedford, MA, U.S.A.). dFBS [dialysed FBS (fetal bovine serum)] was purchased from Sigma (St. Louis, MO, U.S.A.). DN-Src (DN-Src with K296R/Y528F mutations) plasmid and pUSE empty vector were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). FuGENE-6TM transfection reagent was from Roche Diagnostics (Indianapolis, IN, U.S.A.). Geneticin (G418) was purchased from Invitrogen. Recombinant rat TNF α and EGF (epidermal growth factor) were obtained from BD PharMingen International (San Diego, CA, U.S.A.). The ECL (enhanced chemiluminescence) Western Blot detection system was from PerkinElmer (Boston, MA, U.S.A.). DFMO was a gift from ILEX OncologyTM (San Antonio, TX, U.S.A.). Phospho-ERK1/2 (extracellular signal-regulated kinase 1/2), total ERK1/2, phos-

pho-Src (p-Tyr⁴¹⁶), phospho-Akt (p-Ser⁴⁷³), phospho-STAT3 (p-Tyr⁷⁰⁵), cleaved caspase 3 (Asp¹⁷⁵) and rat-specific cleaved PARP [poly(ADP-ribose) polymerase] (Asp²¹⁴) antibodies were from Cell Signaling Technology (Beverly, MA, U.S.A.). p-Tyr⁴¹⁸ Src, p-Tyr⁷⁸⁵ β 3 integrin and p-Ser⁷⁸⁵ β 1 integrin antibodies were obtained from Biosource (Camarillo, CA, U.S.A.). STAT3 and Bcl-2 antibodies were from BD Biosciences (San Diego, CA, U.S.A.). JAK2, phospho-JAK2 (p-Tyr^{1007/1008}) and Src antibodies were purchased from Upstate Biotechnology. PP2 (Src-family tyrosine kinase inhibitor) and AG1478 [EGFR (EGF receptor) kinase inhibitor] were from Calbiochem, EMD Biosciences (La Jolla, CA, U.S.A.) and Biomol International (Plymouth Meeting, PA, U.S.A.) respectively. Protein A/G-agarose conjugate was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The Src tyrosine kinase assay kit was purchased from Upstate Biotechnology. [γ -³²P]ATP was from Amersham Biosciences (Piscataway, NJ, U.S.A.). The Cell Death Detection ELISA Plus kit was from Roche Diagnostics. RGDS (Arg-Gly-Asp-Ser) tetrapeptide was from the American Peptide Company (Sunnyvale, CA, U.S.A.). The IEC-6 cell line (ATCC CRL 1592) was from American Type Culture Collection (Rockville, MD, U.S.A.) at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. [1]. IEC-6 cells originate from intestinal crypt cells as judged by morphological and immunological criteria. They are non-tumorigenic and retain the undifferentiated character of epithelial stem cells. Tests for mycoplasma were always negative. All chemicals were of the highest purity commercially available.

Cell culture

IEC-6 cell stock was maintained in T-150 flasks in a humidified, 37°C incubator in an atmosphere of 10% CO₂. The medium consisted of DMEM (Dulbecco's modified Eagle's medium) with 5% (v/v) heat-inactivated FBS and 10 μ g of insulin and 50 μ g of gentamicin sulphate per ml. The stock flask was passaged weekly, fed three times per week, and passages 15–22 were used. To set up experiments, the cells were trypsinized with 0.05% trypsin and 0.53 mM EDTA and counted by a Beckman Coulter counter (Model Z1). General methods for experiments using IEC-6 cells have already been described [5]. Unless otherwise stated, all experimental set-ups with IEC-6 cells involved a 4 day treatment in control, DFMO and DFMO plus putrescine-containing medium. For short-term DFMO treatment, IEC-6 cells were grown to confluence for 3 days, serum-starved for 24 h and incubated with DFMO-containing medium for the indicated time period. Briefly, IEC-6 cells were plated at 6.24×10^4 cells/cm² in DMEM/5% dFBS (day 0) with or without 5 mM DFMO and DFMO plus 10 μ M putrescine. The cells were fed on day 2. The cells were serum-starved with control, DFMO or DFMO plus putrescine-containing medium for 24 h (on day 3). On day 4, experimental treatments were carried out in the respective serum-free medium and harvesting was performed. Polyamines were depleted by incubation in 5 mM DFMO at 37°C. We have previously reported that maximal polyamine depletion occurs after 4 days of treatment with 5 mM DFMO [5]. Within 6 h of DFMO treatment, putrescine was undetectable, spermidine was absent after 24 h, and 40% of spermine remained after 4 days. One group of cells was given exogenous putrescine (10 μ M) in addition to DFMO. This group acted as a control to indicate that all results were due to the depletion of polyamines and not to DFMO itself.

Transfection

The method for transfection of IEC-6 cells has been previously described [17,25]. Briefly, DN-Src and pUSE (vector) DNA were

prepared using the Qiagen (endotoxin-free) plasmid preparation kit (Valencia, CA, U.S.A.). FuGENE-6™ reagent was mixed with DNA (3 µl/2 µg) in serum-free medium, to a total volume of 100 µl and incubated for 30 min at room temperature (28°C). IEC-6 cells at a relatively early passage were grown to 70–80% confluence in 60 mm dishes. For transfection, the cell monolayer was rinsed with serum-free medium and the DNA/FuGENE-6™ mixture was added dropwise on to the cell monolayers and incubated for a further 12 h at 37°C. For generation of stable lines, the antibiotic-resistant clones were grown to confluence in T-75 flasks. Confluent cultures were trypsinized, and plated at very low density, allowed to form colonies and cloned using cloning cylinders. Selected clones were resistant to 400 µg/ml G418 (Geneticin) and each clone was characterized by Western-blot analysis with Src antibody and apoptosis studies.

Apoptosis studies

Cells were plated (day 0) at a density of 6.24×10^4 cells/cm² in DMEM/5% dFBS with or without DFMO and DFMO plus putrescine, with triplicate samples for each group. Cells were fed on day 2. On day 3, the culture medium was removed and replaced with serum-free medium for another 24 h. On day 4, TNFα (20 ng/ml)/CHX (25 µg/ml) was added to the serum-free medium for 3 h, with the appropriate vehicle added to controls.

Quantitative DNA fragmentation ELISA

Cells were grown in 24-well culture plates for DNA fragmentation ELISA as described previously [6,25]. After treatment, floating cells were discarded and the attached cells were washed twice with DPBS (Dulbecco's PBS). Briefly, cells were lysed and centrifuged to remove the nuclei. An aliquot of the nuclei-free supernatant was placed in streptavidin-coated wells and incubated with anti-histone-biotin antibody and anti-DNA peroxidase-conjugated antibody for 2 h at room temperature. After incubation, the sample was removed and the wells were washed three times with incubation buffer (ELISA kit; Roche). After the final wash was removed, 100 µl of the substrate, 2,2'-azino-di[3-ethylbenzthiazolin-sulphonate], was placed in the wells for 20 min at room temperature. The absorbance was read at 405 nm using a plate reader. Results were expressed as absorbance at 405 nm · min⁻¹ · mg of protein⁻¹.

In vitro c-Src tyrosine kinase assay

Src activity was measured using an *in vitro* kinase assay kit from Upstate Biotechnology as described before [26]. Briefly, confluent IEC-6 monolayers were lysed in RIPA buffer containing 50 mM Tris/HCl (pH 7.4), 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM PMSF, 1 mM NaF, 1 mM Na₃VO₄ and protease inhibitors. Equal amounts of protein (200 µg) were immunoprecipitated with Src antibody and 30 µl of Protein A/G-agarose slurry. Immunoprecipitates were washed three times in RIPA buffer. Beads were resuspended in 60 µl of assay mixture containing 150 µM Src substrate peptide (KVEKIGEGTYGVVYK) in Src reaction buffer (100 mM Tris/HCl, pH 7.2, 125 mM MgCl₂, 25 mM MnCl₂, 2 mM EGTA, 250 µM sodium orthovanadate and 2 mM dithiothreitol), 50 µM ATP and 1 µCi of [γ -³²P]ATP at 30°C for 15 min. Reaction was stopped by keeping the reaction tubes in the ice bath. A 20 µl reaction aliquot was spotted on to P81 Whatman filter discs. Discs were washed three times in 0.75% phosphoric acid. Radioactivity of air-dried discs was counted in a liquid-scintillation counter, Beckman LS500TA (Beckman Coulter, Fullerton, CA, U.S.A.). Activity was expressed as pmol of phosphate incorporated into

substrate per minute and presented as units/mg of protein. Activity present in corresponding immune complexes prepared using pre-immune mouse IgG was subtracted from the activity in anti-c-Src immune complexes. Data points were calculated and expressed as activity in units per mg of protein.

Western-blot analysis

Cell monolayers were washed with ice-cold DPBS and lysates were prepared as described earlier [27]. Supernatants (25–50 µg of protein) from cell extracts were trichloroacetic acid-precipitated and dissolved in 1× SDS sample buffer (62.5 mM Tris/HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue and 5% 2-mercaptoethanol) for 5 min and separated by SDS/10–15%-PAGE. Proteins were transferred overnight on to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) and probed with the indicated antibodies overnight at 4°C in TBS (Tris-buffered saline) buffer containing 0.1% Tween 20 and 5% (w/v) non-fat dry milk (blotting grade, Bio-Rad). BSA solution (5%) in TBST buffer [10 mM Tris, 0.9% (w/v) NaCl and 0.1% Tween 20 detergent with a final pH adjusted to 8.0] was used for blocking for Western blots with anti-phosphotyrosine antibody. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h and the immunocomplexes were visualized by the ECL detection system (PerkinElmer). Blots were stripped and probed with the indicated antibodies to determine equal loading of the samples.

Densitometry and statistical analysis

Quantification of Western blots was carried out using Image J 1.34s software (NIH, Bethesda, MD, U.S.A.) and expressed as percentage of untreated control as described earlier [25]. All data are expressed as means ± S.E.M. Experiments were repeated three times, with triplicate samples for each. ANOVA and appropriate post-hoc testing determined the significance of the differences between means. Values of $P < 0.05$ were regarded as significant.

RESULTS

Polyamine depletion and Src activation

Polyamine depletion stimulated Src Tyr⁴¹⁸ phosphorylation, and phosphorylation was prevented when cells were grown with DFMO plus exogenous putrescine (Figure 1A). Immunoprecipitation of Src followed by Western-blot analysis with phosphotyrosine antibody also showed increased levels of tyrosine-phosphorylated Src (results not shown). Additional *in vitro* c-Src kinase assays showed an activity of 29.27 ± 2.76 units/mg of protein in control cells, which increased to 72.36 ± 2.73 units/mg of protein in polyamine-depleted cells ($P < 0.05$; mean ± S.E.M., $n = 6$). Exogenous putrescine blocked the activation to 31.85 ± 3.04 units/mg of protein, which is similar to control cells. Src protein levels were the same in all three groups.

Src inactivation and apoptosis

Our earlier observations showed that significantly higher proportions of cells detached from plates with significant increases in caspase 3 activation after 6–9 h of treatment with TNFα/CHX [6]. We determined the level of Src p-Tyr⁴¹⁶ (active Src) during cell detachment and apoptosis (Figure 1B). Src p-Tyr⁴¹⁶ levels decreased significantly in response to TNFα/CHX compared with untreated cells in a time-dependent manner. Total Src protein also decreased with time, indicating progression of apoptosis and

Figure 1 Tyrosine phosphorylation and activation of Src by polyamine depletion

(A) Western-blot analysis of equal amounts of whole cell extracts using antibody specific for p-Tyr⁴¹⁸ Src. Blots were reprobed for total Src protein. Representative blots from three observations are shown. (B) Time-dependent Src p-Tyr⁴¹⁶ activation in response to TNFα treatment. IEC-6 cells were grown as described in the Experimental section and treated with TNFα/CHX for 6 and 9 h. Western-blot analyses are shown with antibodies recognizing either total or phosphorylated Src. Membranes were stripped and probed with anti-actin antibody to confirm equal loading of samples. Representative blots from three independent observations are shown. PUT, putrescine.

cleavage of Src protein by activated caspases (results not shown). Cells grown in the presence of 5 mM DFMO had increased Src p-Tyr⁴¹⁶ phosphorylation, which was sustained throughout the entire time course of TNFα/CHX treatment. Constant Src protein levels in polyamine-depleted cells indicate a lack of caspase activation due to the inhibition of apoptosis. The changes observed in cells incubated with DFMO plus exogenous putrescine were qualitatively identical with those in control cells (Figure 1B).

Inhibition of Src with PP2 increases apoptosis and prevents the activation of JAK, STAT3 and Akt

The pharmacological Src-family kinase inhibitor PP2 was used to block enzyme activity. PP2 is a widely used inhibitor of Src and has been shown to be effective in many cell types including colon cancer cell lines [28], human colon cancer cells [29] and normal hepatocytes [30]. PP2 prevented the phosphorylation and activation of Src normally seen in the polyamine-depleted cells (results not shown). In control cells, PP2 significantly increased apoptosis over that produced by TNFα/CHX alone (Figure 2A). In control cells treated with TNFα/CHX, the inhibition of Src significantly increased DNA fragmentation over the amount seen

Figure 2 Src kinase inhibitor PP2 reverses the protective effect of DFMO

(A) Serum-starved IEC-6 cells were pretreated with 20 μM PP2 for 1 h followed by TNFα/CHX for 3 h. DNA fragmentation was assayed as described above. **P* < 0.005 compared with respective untreated group, ***P* < 0.005 compared with TNFα/CHX-treated group and †*P* < 0.005 compared with respective TNFα/CHX-treated control and DFMO plus putrescine grown cells (mean ± S.E.M., *n* = 6). (B) PP2 blocks JAK2, STAT3 and Akt phosphorylation. IEC-6 cells were pretreated with or without 20 μM PP2 for 1 h followed by TNFα/CHX treatment for 3 h. Western-blot analyses are shown using phospho-JAK2, phospho-STAT3 and phospho-Akt-specific antibodies. Representative blots from three independent observations are shown.

with TNFα/CHX alone. In cells grown in the presence of DFMO, the level of apoptosis in response to TNFα/CHX was significantly less compared with that seen in control cells, which is consistent with our previous observations [6,25,27]. In response to PP2 alone, apoptosis significantly increased to the level seen with TNFα/CHX plus PP2 in control cells. The inhibition of Src completely removed the protection from TNFα/CHX normally seen following polyamine depletion, for the level of apoptosis in the DFMO group in response to TNFα/CHX following inhibition with PP2 was increased over that seen in control cells after TNFα/CHX (Figure 2A). Cells grown in the presence of DFMO plus putrescine had responses identical with control cells.

In polyamine-depleted cells, JAK2, STAT3 and Akt were constitutively active, while there was little or no activity in control cells (Figure 2B). TNFα/CHX activated JAK, STAT3 and Akt in control cells, and the activation of all three proteins was inhibited by PP2. In polyamine-depleted cells, TNFα/CHX increased the phosphorylation of the three proteins somewhat over constitutive levels, and PP2 significantly inhibited activation in each case (Figure 2B). In all groups, the levels of all three proteins remained relatively constant. The addition of putrescine prevented all the effects of DFMO (results not shown).

Figure 3 Dominant-negative mutant of Src blocks the protective effect of DFMO and decreases Bcl-2 protein level

(A) Empty vector (EV) and DN-Src cells were grown as described in the Experimental section and treated with TNF α /CHX for 3 h. DNA fragmentation was measured by a cell death detection ELISA method. * $P < 0.005$ compared with untreated cells. † $P < 0.005$ compared with respective TNF α /CHX-treated vector cells. # $P < 0.005$ compared with respective untreated vector cells (mean $\pm$ S.E.M., $n = 6$). PUT, putrescine. (B) Expression of DN-Src induces caspase 3 and PARP cleavage. Empty vector (EV) and DN-Src cells were treated with TNF α /CHX for 3 h. Cell lysates were probed with an antibody specific for active caspase 3 and a cleaved PARP (Asp²¹⁴) rat-specific antibody. Representative blots from three independent observations are shown. (C) Western-blot analysis of whole cell lysates of empty vector and DN-Src-expressing cells with Bcl-2-specific antibody. Representative blots from three independent observations are shown.

Expression of DN-Src inhibits Src, prevents activation of JAK, STAT3 and Akt and increases apoptosis in polyamine-depleted IEC-6 cells

Cells expressing DN-Src had less phosphorylated and activated Src than those transfected with empty vector. Src protein was also increased in the DN-cells, for the Src antibody we used recognizes the recombinant as well as endogenous Src protein (results not shown). In cells transfected with empty vector, DNA fragmentation in response to TNF α /CHX in polyamine-depleted cells was approx. 25 % of that in control cells (Figure 3A). Spontaneous apoptosis was elevated significantly in cells with DN-Src, and the amount of apoptosis in response to TNF α /CHX was more than double when compared with that seen in the vector cells. In cells transfected with DN-Src, there was no protective effect of polyamine depletion, and the degrees of apoptosis in all three groups were approximately equal. We also monitored apoptosis by measuring the levels of active caspase 3 and cleaved PARP, a substrate for caspase 3 (Figure 3B). In control cells, TNF α /CHX caused the activation of caspase 3 and cleavage

of PARP. These effects were enhanced in cells expressing DN-Src. Polyamine depletion prevented the increases in caspase 3 activity and its PARP cleavage product in vector cells. Both caspase 3 and its cleavage product were, however, induced by TNF α /CHX in DFMO-treated DN-Src-expressing cells. Cells incubated with putrescine and DFMO produced the same results as the control group (Figure 3B). Actin was used as a loading control and did not change (results not shown). Transfection with DN-Src mimicked the effects of PP2 on STAT3, JAK and Akt phosphorylation (see Supplementary material at <http://www.biochemJ.org/bj/397/bj3970437add.htm>). In each case, activation caused by DFMO and polyamine depletion was prevented in cells expressing DN-Src. These results together with PP2 results provide strong evidence that Src is responsible for the activation of JAK, STAT3 and Akt and the subsequent protection from apoptosis. This conclusion is further strengthened by the finding that DN-Src prevented DFMO-mediated increase in the levels of Bcl-2 protein as seen in empty vector-transfected cells (Figure 3C).

Effects of short-term treatment of DFMO on Src activation

Following 4 days of polyamine depletion, Src (Figure 1), STAT3 and NF- κ B are constitutively active and contribute to the resistance to apoptosis in these cells [25,27]. In our previous studies, both transcription factors were activated within 1 h of treatment with DFMO, and in both cases activation was prevented by the inclusion of an exogenous polyamine in the incubation medium along with DFMO [31,32]. Thus activation is due to the decrease in free polyamines caused by abrupt cessation of ODC activity and polyamine synthesis. Since Src is known to activate STAT3 and NF- κ B, we determined whether Src activation precedes the activation of these transcription factors following short-term treatment with DFMO. Figure 4(A) depicts the time course for the activation of Src following DFMO treatment. The actual Src kinase activity increased significantly from 100 % in control cells to 189 ± 10.77 % at the 30 min time point of DFMO treatment and remained elevated for the 6 h duration of the study ($P < 0.05$; mean $\pm$ S.E.M., $n = 6$) (Figure 4A). Similar to our previous studies, addition of exogenous putrescine to the DFMO-containing medium blocked DFMO-mediated Src kinase activity to 103.80 ± 1.48 % after 30 min treatment (results not shown). Pre-incubation with pharmacological Src kinase inhibitor PP2 completely blocked DFMO-mediated Src activation (Figure 4C). In order to determine other downstream signalling events of Src activation following short-term DFMO treatment, we investigated STAT3 and ERK1/2 activation. Activation of Src, STAT3 and ERK1/2 was followed by determining the levels of Tyr⁴¹⁸, Tyr⁷⁰⁵ and Thr²⁰²/Tyr²⁰⁴ phosphorylation respectively. Src and ERK1/2 activation occurred within 30 min of the addition of DFMO, and preceded STAT3 activation at 1 h (Figure 4B). Similar to previous experimental results, total protein levels of Src, STAT3 and ERK1/2 remained unchanged throughout the entire duration of DFMO treatment (results not shown).

Src activation in response to EGF and DFMO

We compared the level of DFMO-mediated Src activation with EGF. Results presented in Figure 5 show Src p-Tyr⁴¹⁸ and ERK1/2 phosphorylation in confluent intestinal epithelial cells when treated with DFMO or EGF for 30 min. The extent of Src p-Tyr⁴¹⁸ phosphorylation was similar in both cases (Figure 5A). As with ERK1/2, EGF-mediated activation was higher when compared with DFMO treatment. Pretreatment with AG1478, a potent pharmacological inhibitor of EGFR kinase [33], significantly blocked EGF-mediated Src activation but had no significant

Figure 4 Time course of tyrosine phosphorylation of Src and STAT3 after DFMO treatment

(A) Lysates from IEC-6 cells at times indicated following incubation with 5 mM DFMO in serum-free medium were immunoprecipitated with c-Src-specific antibody and kinase activity was determined as described in the Experimental section. * $P < 0.05$ compared with untreated cells (mean $\pm$ S.E.M., $n = 6$). (B) Lysates prepared from IEC-6 cells at times indicated after DFMO addition were immunoblotted with p-Tyr⁴¹⁸ Src, p-Tyr⁷⁰⁵ STAT3 and phospho-ERK1/2-specific antibodies. Representative blots from three independent observations are shown. (C) Lysates from IEC-6 cells pretreated with or without 20 μ M PP2 for 1 h followed by 30 min treatment with 5 mM DFMO were analysed for p-Tyr⁴¹⁸ Src by Western blotting. Membranes were reprobed for total Src protein. Representative blots from three independent observations are shown.

effect on the DFMO response. However, ERK1/2 phosphorylation induced by either DFMO or EGF was completely blocked by AG1478, indicating that inhibition of polyamine synthesis increases Src and ERK activity through different mechanisms.

Addition of exogenous polyamines blocked the activation of Src and ERK in response to DFMO but had no effect on EGF-mediated Src and ERK1/2 phosphorylation (Figure 5B). Our results indicate that exogenous polyamines can selectively block the effect of ODC inhibition but have no effect on EGF-mediated signalling.

Polyamines and integrin-mediated Src activation

Attachment of fibronectin and α v β 3 is known to stimulate phosphorylation of Src and FAK [24,34]. Since DFMO activated

Figure 5 EGF- and DFMO-mediated Src and ERK1/2 activation

(A) Confluent IEC-6 cells were incubated at 37°C with serum-free medium containing EGF (10 ng/ml) or DFMO (5 mM) for 30 min in the presence and absence of 10 μ M AG1478. Cells were pre-incubated with AG1478 in serum-free medium for 45 min before the addition of DFMO or EGF. Cell lysates were analysed by Western blotting using antibodies specific for p-Tyr⁴¹⁸ Src and phospho-ERK1/2. Representative blots from three independent observations are shown. UT, untreated. (B) Confluent, serum-starved cells were treated with DFMO or EGF for 30 min as mentioned above in the presence and absence of exogenous PUT (10 μ M putrescine), SPD (5 μ M spermidine) and SPM (5 μ M spermine). Cell lysates were analysed by Western blot using antibodies specific for Src p-Tyr⁴¹⁸ and phospho-ERK1/2. Representative blots from three independent observations are shown.

Src, we wanted to evaluate the role of integrin signalling as an upstream event in Src activation. First, in order to determine the consequences of fibronectin and α v β 3 receptor interaction, we seeded IEC-6 cells on fibronectin-coated plates and compared the effect with those on plastic and cells in suspension. A significant increase in p-Tyr⁷⁸⁵ β 3 integrin and p-Tyr⁴¹⁸ Src phosphorylation was observed in cells seeded on fibronectin when compared with plastic or cells in suspension (Figure 6A). Addition of exogenous putrescine or spermine to the fibronectin matrix completely blocked β 3 integrin and Src phosphorylation

Figure 6 Integrin β_3 and Src activation

(A) IEC-6 cells were trypsinized and the suspension was conditioned in serum-free medium for 1 h. Equal numbers of conditioned cells were seeded in plastic- or fibronectin-coated plates containing serum-free medium supplemented with and without putrescine (PUT) or spermine (SPM), or left in suspension (Susp) for 2 h. Floating cells were removed by washing with serum-free DMEM, and attached cells were lysed. Cell lysates were analysed with p-Tyr⁴¹⁸ Src and p-Tyr⁷⁸⁵ integrin β_3 antibodies. Membranes were re-probed for total Src and integrin β_3 . Representative blots from three independent observations are shown. (B) Serum-starved cells grown under control or DFMO conditions were incubated with or without RGDS peptide (5 mM) for 2 h. Western-blot analysis was performed using antibodies specific for integrin β_3 p-Tyr⁷⁸⁵ and total β_3 antibody. Densitometric analyses were performed as described in the Experimental section and expressed as percentage of untreated control. Representative blots from three independent observations are shown. (C) Cells were grown and treated with the indicated concentrations of RGDS peptide as described above. Cell lysates were probed with p-Tyr⁴¹⁸ Src and p-Ser⁴⁷³ Akt antibodies. Representative blots from three independent observations are shown.

(Figure 6A). Total Src and β_3 integrin protein levels did not change. Exogenous polyamines also decreased cell attachment on plastic and fibronectin-coated plates (results not shown). Polyamine depletion increased p-Tyr⁷⁸⁵ β_3 phosphorylation when compared with cells grown under control conditions (Figure 6B). Our results indicate that polyamines might interfere with integrin binding or clustering in response to ECM (extracellular matrix)–receptor interaction as ‘outside-in signalling’ or they could also prevent the recruitment of Src to the membrane and its subsequent phosphorylation induced by β_3 integrin (‘inside-out signalling’).

A recent study shows Src kinase activation by direct interaction with cytoplasmic domain of integrin β_3 [23]. Since integrin engagement by fibronectin activated Src, we tested Src activation in the presence of integrin receptor inhibitor RGDS peptide. Pre-

Figure 7 RGDS peptide and DNA fragmentation

(A) Serum-starved confluent IEC-6 cell monolayers were treated with 0.5, 1 and 5 mM RGDS peptide for 2 h followed by treatment with TNF α /CHX for 3 h. DNA fragmentation was measured as described earlier. * P < 0.005 compared with untreated cells. † P < 0.005 compared with TNF α /CHX-treated group (mean $\pm$ S.E.M., n = 6). (B) IEC-6 cells grown in control or DFMO-containing medium were treated with 5 mM RGDS for 2 h followed by TNF α /CHX for 3 h. DNA fragmentation was measured as described earlier. * P < 0.005 compared with untreated cells. ** P < 0.005 compared with TNF α /CHX-treated group (mean $\pm$ S.E.M., n = 6). † P < 0.005 compared with control cells treated with TNF α /CHX.

treatment with RGDS significantly blocked integrin β_3 p-Tyr⁷⁸⁵ phosphorylation in control and DFMO-treated cells (Figure 6B). Total integrin β_3 protein level was unaltered. Concurrently with the inhibition of β_3 phosphorylation, RGDS inhibited p-Tyr⁴¹⁸ Src and p-Ser⁴⁷³ Akt activation in a dose-dependent manner (Figure 6C). Total Src and Akt protein levels did not change during this treatment (results not shown). RGDS significantly increased apoptosis in a dose-dependent manner in the absence of TNF α /CHX (Figure 7A). Pretreatment of the cells with RGDS further potentiated the apoptotic effect of TNF α /CHX. In cells grown in the presence of DFMO, the apoptosis observed with TNF α /CHX was significantly low, which is consistent with our previous observations [6,25,27]. RGDS inhibited Src activation and completely reversed the protective effect of DFMO, for the level of apoptosis was more than that observed in control cells treated with TNF α /CHX (Figure 7B). These results support a model of Src activation by β_3 integrin in response to polyamine depletion and confirm the role of activated Src in protecting cells from apoptosis induced by TNF α /CHX.

Figure 8 Integrin β_3 activation after polyamine depletion and protection from apoptosis

(A) Western-blot analysis with p-Tyr⁷⁸⁵ β_3 and p-Ser⁷⁸⁵ β_1 antibodies. Membranes were probed with β_3 - and β_1 -specific antibodies. (B) IEC-6 monolayers were treated with either DFMO or DFMO plus 10 μ M putrescine (PUT) containing medium for 30 min, and equal amounts of proteins were immunoprecipitated (IP) with β_3 -specific antibody followed by Western-blot analysis (WB) with p-Tyr⁷⁸⁵ β_3 antibody. Membranes were re-probed with the integrin β_3 antibody. Representative blots from three observations are shown. Densitometric analyses of Western blots were carried out as described in the Experimental section. (C) IEC-6 cells were grown on plastic- and fibronectin-coated plates for 24 h followed by serum starvation for 24 h prior to TNF α /CHX treatment for 3 h. DNA fragmentation was measured as described earlier. * $P < 0.005$ compared with TNF α /CHX-treated group (mean $\pm$ S.E.M., $n = 6$).

Polyamines, integrin β_3 and apoptosis

Clustering of integrin receptors leads to phosphorylation of Tyr⁷⁸⁵ on integrin β_3 [35]. Polyamine depletion increased β_3 Tyr⁷⁸⁵ phosphorylation when compared with control cells (Figure 8A). However, β_1 Ser⁷⁸⁵ phosphorylation did not significantly change after polyamine depletion, indicating a specific role for polyamines in the activation of integrins (Figure 8A). Addition of exogenous putrescine to the DFMO medium prevented the increase

in β_3 integrin phosphorylation. No changes in β_1 or β_3 protein levels were observed after polyamine depletion. Since short-term DFMO treatment increased Src activation, we examined whether β_3 integrin was activated following inhibition of ODC activity. Similar to 4 days of polyamine depletion, treatment of cells with DFMO for 30 min increased β_3 p-Tyr⁷⁸⁵ levels compared with control cells (Figure 8B). Consistent with our results described in Figure 8(A), addition of putrescine completely blocked DFMO-mediated increase in integrin phosphorylation (Figure 8B), indicating that loss of cellular putrescine immediately causes β_3 phosphorylation, which in turn activates Src and thereby activates Akt and STAT3.

Src activation following 4 days DFMO treatment conferred protection from apoptosis. Inhibition of Src by PP2, a DN-Src construct or the integrin β_3 antagonist RGDS, sensitized polyamine-depleted cells to apoptosis. Therefore Src activation by fibronectin should at least partly protect cells from apoptosis. Treatment of cells with fibronectin-coated plates significantly protected them from TNF α /CHX-induced apoptosis, indicating a role of activated integrin β_3 and Src kinase in mediating cell survival (Figure 8C).

DISCUSSION

A wide range of stimuli that govern cell survival regulate signalling events which act through cell-surface receptors and transmit inward signals via tyrosine and serine/threonine kinases. Integrins, in their role as principal cellular receptors for the ECM, mediate cell adhesion and regulate cell morphology. The activation of v-Src [viral Src oncogene isolated from RSV (Rous sarcoma virus)] or Ha-Ras [family of retrovirus-associated DNA sequences (ras) originally isolated from Harvey murine sarcoma virus] or the integrin-mediated attachment to RGD (Arg-Gly-Asp)-containing ligands, but not by soluble RGD peptides, suppressed anoikis [36]. Meredith et al. [37] showed that attachment to substrate without integrin engagement induced apoptosis, while integrin-mediated attachment led to cell survival. Integrin-mediated survival signalling regulates several factors, including Bcl-2 family protein expression [38] and activation of PI3K/Akt [39]. Since activation of Src-mediated survival pathways in polyamine-depleted cells resembled integrin-mediated responses, and polyamines stabilized membrane structure, we predicted that polyamines might regulate membrane proximal integrin-mediated signalling events leading to the activation of Src and inhibition of apoptosis. The increased phosphorylation of β_3 integrin and Src during attachment and the fact that fibronectin further increased their activation imply that the activation of integrin β_3 might be responsible for the activation of Src in these cells (Figure 6A). Exogenous polyamines (putrescine and spermine) added to plates during attachment significantly prevented the activation of both integrin β_3 and Src. The inhibition of integrin β_3 , Src and Akt activation by RGDS (Figures 6B and 6C), a soluble peptide derived from the integrin $\alpha v \beta_3$ binding region of fibronectin, strongly suggests the involvement of integrins in the regulation of Src. RGDS peptide completely eliminated the protection conferred by the depletion of polyamines (Figure 7A and 7B). RGDS peptide induces apoptosis in human endothelial cells [40] and rat cardiomyocytes [41]. The interference with integrin-mediated signalling by RGDS increased apoptosis in the absence of TNF α /CHX, indicating the significance of integrin-mediated adherence in cell survival.

The question remains as to how polyamine depletion activates Src. Src family members possess several protein domains. The N-terminal end of the molecule contains a membrane-targeting

signal, which is followed in order by a unique sequence, SH3 (Src homology 3) and SH2 domains, the catalytic domain and a negative regulatory tail [42]. We found that short-term treatment with DFMO also elicited responses, which were similar to those reported for the polyamine-depleted cells (4 day DFMO treatment) (Figures 2B and 4B). We compared the responses of cells after short-term treatment with DFMO with growth factor (EGF) to ascertain that the DFMO-mediated effects are specific and are due to the modulation of polyamine levels. EGF- and DFMO-induced ERK activation can be blocked by EGFR tyrosine kinase inhibitor (AG1478). However, AG1478 could block EGF-induced Src activation but had no effect on DFMO-induced Src activation (Figure 5A). Furthermore, exogenous addition of putrescine, spermidine and spermine along with DFMO significantly decreased the activation of Src and ERKs but had no effect on EGF-induced activation of Src and ERKs (Figure 5B). Thus polyamines and EGF appear to induce Src activation through EGFR-independent and -dependent mechanisms respectively. Interestingly, these results indicate that EGFR activation is a consequence of polyamine depletion. Or else it suggests the trans-activation of EGFR either directly by Src or by polyamine-sensitive membrane receptors. Arias-Salgado et al. [23] have shown that Src binds constitutively to $\beta 3$ integrin via its SH3 domain and C-terminal cytoplasmic tail of the integrin. Clustering of $\beta 3$ integrin activates Src *in vivo*, inducing phosphorylation of Tyr⁴¹⁸ [23]. In polyamine-depleted cells, integrin $\beta 3$ is clustered, which recruits and activates c-Src kinase and associates in a complex involving integrins and other adaptor proteins, leading to phosphorylation of the EGFR. Moro et al. [43] have shown that integrin-mediated adhesion requires a macromolecular complex involving c-Src and p130Cas and consequently leads to the activation of EGFR. Polyamines could directly prevent integrin clustering leading to inhibition of EGFR phosphorylation or could inhibit the adhesion-dependent macromolecular complex involving the cytoplasmic tail of integrin and Src required for the phosphorylation of the EGFR.

The interactions of polyamines with proteins are numerous and of profound importance; for instance, they bind to a great variety of receptors [44]. Polyamines also stabilize the secondary and the tertiary structures of macromolecules by electrostatic interactions. In addition, they induce conformational changes and thereby preserve or alter physical and biological properties of proteins and polyanionic molecules. Besides the formation of ionic bonds, polyamines are also functionally active in programmed cell death. Spermine prevents endonuclease activation by stabilizing it in an inactive conformation [45]. Putrescine, spermidine and spermine contain two, three and four amine groups respectively. These amine groups have pK values above 9 and are almost completely protonated at physiological pH. As such, polyamines bind strongly to negatively charged molecules, particularly proteins and nucleic acids. Since the cations in polyamines are not point charges, but are fixed along a flexible carbon chain, they are able to interact with macromolecules in structurally specific ways [46]. Polyamines could, theoretically, interact directly with Src keeping it in the inactive state or, on the other hand, could act to prevent integrin clustering. Polyamine depletion for 4 days or short-term (30 min) DFMO treatment increased $\beta 3$ Tyr⁷⁸⁵ phosphorylation, whereas phosphorylation of integrin $\beta 1$ was unaltered (Figures 8A and 8B). Increased $\beta 3$ integrin phosphorylation could recruit Src kinase to the membrane and enhance integrin–Src association in polyamine-depleted cells. Taken together, it appears that polyamines might modulate Src-mediated survival through $\beta 3$ integrin in IEC-6 cells.

Of particular interest was the activation of Src within 30 min of treating cells with DFMO (Figures 4A and 4B). This requires

revisiting the concept of 'polyamine depletion'. In IEC-6 cells, putrescine is reduced approx. 50 % 1 h after administering DFMO and reaches undetectable levels by 6 h. Spermidine and spermine do not begin to decline until 6 h. Spermidine is undetectable at 48 h, and spermine is only reduced by 60 % by 4 days [5]. These results indicate that Src activation is not caused by a general depletion of polyamines. Instead, we believe that there is only a small pool of free or available polyamines within the cell. This pool, consisting primarily of putrescine, is rapidly converted into spermidine and depleted following the inhibition of ODC. Since almost all intracellular polyamines must be bound and unavailable for biological processes, the depletion of free polyamines and those in equilibrium with it is sensed rapidly by the cell. This view of maintaining cellular polyamine equilibrium is supported by the finding that ODC activity reaches a peak within 2–4 h of treating cells with serum or growth factors [47]. It is obvious that cellular polyamines are not available to participate in the proliferative response and new ones must be synthesized.

Our results present evidence that DFMO and subsequent polyamine depletion activate Src which in turn results in the inhibition of apoptosis by inducing Akt and JAK activity and the subsequent activation of the transcription factors NF- κ B and STAT3 respectively. Previous work showed that NF- κ B DNA-binding activity increased within 1 h after treating IEC-6 cells with DFMO [31]. We have recently shown that the protection from apoptosis by activated Akt in polyamine-depleted cells is dependent on the nuclear translocation and activation of NF- κ B [27].

In the present study, STAT3 was phosphorylated on Tyr⁷⁰⁵ and activated within 1 h of treatment with DFMO. Src activation preceded the activation observed for STAT3 (Figures 4A and 4B) and reported for NF- κ B [31]. In previous studies, we found that inhibition of ODC rapidly induced STAT3 activation [32]. Inhibition of STAT3 increased the sensitivity of polyamine-depleted cells to apoptosis [25]. Src remained constitutively activated in the presence of DFMO as evidenced by tyrosine phosphorylation and kinase activity (Figure 1A). These responses were prevented by exogenous polyamines (Figure 1A). Src kinases play central roles in regulating cell survival [20–22], and the survival signals are usually mediated through the Akt–NF- κ B or STAT3 pathways [20,21]. In intestinal epithelial cells and colon cancer cells, for example, increased activity of Src has been shown to delay anoikis [22,28]. Inhibition of Src reduced STAT3 activity and induced apoptosis of human non-small cell carcinoma cells [48]. We found that the inhibition of Src either with PP2 or cells expressing DN-Src increased apoptosis of control cells both in the absence and presence of TNF α /CHX and prevented the protective effect of polyamine depletion (Figures 2A and 3A). TNF α /CHX simultaneously activates pro- and anti-apoptotic pathways; however, the presence of CHX prevents synthesis of short-lived antiapoptotic proteins such as cIAP2 (cellular inhibitor of apoptosis protein 2) and Bcl-2 and enhances proapoptotic signalling. Polyamine-depleted cells already had increased levels of Bcl-2 and cIAP2 due to sustained activation of Akt-mediated NF- κ B and STAT3 prior to TNF α /CHX treatment [25,27]. Thus the initial survival response to TNF α /CHX treatment activated Akt and STAT3, which gradually decreased in a time-dependent manner in control cells, leading to apoptosis [25,27]. In the same experiment, both PP2- and DN-Src-expressing cells decreased the activation of JAK, STAT3 and Akt in control cells and prevented their activation in polyamine-depleted cells (Figure 2B and Supplementary material). Although polyamine-depleted DN-Src cells had DNA fragmentation equivalent to control DN-Src cells, caspase 3 activation was significantly lower in these cells (Figure 3B). Increased DNA fragmentation observed in polyamine-depleted DN-Src cells may result from the activation of

both caspase 3 and caspase 6 in these cells [6]. Bcl-2 protein increased in vector cells that were incubated with DFMO, and this increase was totally prevented in the DN-Src-transfected cells (Figure 3C). Thus these results established that Src is activated by DFMO and subsequent polyamine depletion and that it accounts for the resistance to apoptosis seen in polyamine-depleted intestinal epithelial cell by activating the JAK–STAT3 and Akt–NF- κ B pathways.

In conclusion, the results presented show that the polyamines regulate integrin β 3-dependent activation of Src by its phosphorylation at Tyr⁴¹⁸ and that it in turn activates the Akt–NF- κ B and the JAK–STAT3 signalling pathways. These transcription factors direct the synthesis of proteins that increase the resistance of polyamine-depleted cells to apoptosis.

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REFERENCES

- Quaroni, A., Wands, J., Trelstad, R. L. and Isselbacher, K. J. (1979) Epithelioid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. *J. Cell Biol.* **80**, 248–265
- Potten, C. S., Wilson, J. W. and Booth, C. (1997) Regulation and significance of apoptosis in the stem cells of the gastrointestinal epithelium. *Stem Cells* **15**, 82–93
- Potten, C. S. (1997) Epithelial cell growth and differentiation 2: intestinal apoptosis. *Am. J. Physiol.* **273**, G253–G257
- Deng, W., Viar, M. J. and Johnson, L. R. (2005) Polyamine depletion inhibits irradiation-induced apoptosis in intestinal epithelia. *Am. J. Physiol. Gastrointest. Liver Physiol.* **289**, G599–G606
- Ray, R. M., Viar, M. J., Yuan, Q. and Johnson, L. R. (2000) Polyamine depletion delays apoptosis of rat intestinal epithelial cells. *Am. J. Physiol. Cell Physiol.* **278**, C480–C489
- Bhattacharya, S., Ray, R. M., Viar, M. J. and Johnson, L. R. (2003) Polyamines are required for activation of c-Jun NH₂-terminal kinase and apoptosis in response to TNF- α in IEC-6 cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **285**, G980–G991
- Hsu, H., Shu, H. B., Pan, M. G. and Goeddel, D. V. (1996) TRADD–TRAF2 and TRADD–FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* **84**, 299–309
- Yuan, Q., Ray, R. M. and Johnson, L. R. (2002) Polyamine depletion prevents camptothecin-induced apoptosis by inhibiting the release of cytochrome c. *Am. J. Physiol. Cell Physiol.* **282**, C1290–C1297
- Deveraux, Q. L. and Reed, J. C. (1999) IAP family proteins—suppressors of apoptosis. *Genes Dev.* **13**, 239–252
- Beg, A. A. and Baltimore, D. (1996) An essential role for NF- κ B in preventing TNF- α -induced cell death. *Science* **274**, 782–784
- Bowman, T., Garcia, R., Turkson, J. and Jove, R. (2000) STATs in oncogenesis. *Oncogene* **19**, 2474–2488
- Pegg, A. E. and McCann, P. P. (1982) Polyamine metabolism and function. *Am. J. Physiol.* **243**, C212–C221
- Pegg, A. E. (1986) Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.* **234**, 249–262
- Tabor, C. W. and Tabor, H. (1984) Polyamines. *Annu. Rev. Biochem.* **53**, 749–790
- Russell, D. H. (1985) Ornithine decarboxylase: a key regulatory enzyme in normal and neoplastic growth. *Drug Metab. Rev.* **16**, 1–88
- Pegg, A. E., McGovern, K. A. and Wiest, L. (1987) Decarboxylation of α -difluoromethylornithine by ornithine decarboxylase. *Biochem. J.* **241**, 305–307
- Ray, R. M., McCormack, S. A., Covington, C., Viar, M. J., Zheng, Y. and Johnson, L. R. (2003) The requirement for polyamines for intestinal epithelial cell migration is mediated through Rac1. *J. Biol. Chem.* **278**, 13039–13046
- Schipper, R. G., Penning, L. C. and Verhofstad, A. A. (2000) Involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors? *Semin. Cancer Biol.* **10**, 55–68
- Frame, M. C. (2002) Src in cancer: deregulation and consequences for cell behaviour. *Biochim. Biophys. Acta* **1602**, 114–130
- Murillo, M. M., del Castillo, G., Sanchez, A., Fernandez, M. and Fabregat, I. (2005) Involvement of EGF receptor and c-Src in the survival signals induced by TGF- β 1 in hepatocytes. *Oncogene* **24**, 4580–4587
- Proietti, C., Salatino, M., Rosenblit, C., Carnevale, R., Pecci, A., Kornblith, A. R., Molinolo, A. A., Frahur, I., Charreau, E. H., Schillaci, R. and Elizalde, P. V. (2005) Progesterone induce transcriptional activation of signal transducer and activator of transcription 3 (Stat3) via a Jak- and Src-dependent mechanism in breast cancer cells. *Mol. Cell. Biol.* **25**, 4826–4840
- Coll, M. L., Rosen, K., Ladedo, V. and Filmus, J. (2002) Increased Bcl-xL expression mediates v-Src-induced resistance to anoikis in intestinal epithelial cells. *Oncogene* **21**, 2908–2913
- Arias-Salgado, E. G., Lizano, S., Sarkar, S., Brugge, J. S., Ginsberg, M. H. and Shattil, S. J. (2003) Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13298–13302
- Shattil, S. J. (2005) Integrins and Src: dynamic duo of adhesion signaling. *Trends Cell Biol.* **15**, 399–403
- Bhattacharya, S., Ray, R. M. and Johnson, L. R. (2005) STAT3-mediated transcription of Bcl-2, Mcl-1 and c-IAP2 prevents apoptosis in polyamine-depleted cells. *Biochem. J.* **392**, 335–344
- Nishida, M., Maruyama, Y., Tanaka, R., Kontani, K., Nagao, T. and Kurose, H. (2000) G α_i and G α_o are target proteins of reactive oxygen species. *Nature (London)* **408**, 492–495
- Bhattacharya, S., Ray, R. M. and Johnson, L. R. (2005) Decreased apoptosis in polyamine depleted IEC-6 cells depends on Akt-mediated NF- κ B activation but not GSK3 β activity. *Apoptosis* **10**, 759–776
- Golubovskaya, V. M., Gross, S., Kaur, A. S., Wilson, R. I., Xu, L. H., Yang, X. H. and Cance, W. G. (2003) Simultaneous inhibition of focal adhesion kinase and SRC enhances detachment and apoptosis in colon cancer cell lines. *Mol. Cancer Res.* **1**, 755–764
- Griffiths, G. J., Koh, M. Y., Burnton, V. G., Cawthorne, C., Reeves, N. A., Greaves, M., Tilley, M. J., Pearson, D. G., Ottley, C. J., Workman, P. et al. (2004) Expression of kinase-defective mutants of c-Src in human metastatic colon cancer cells decreases Bcl-xL and increases oxaliplatin- and Fas-induced apoptosis. *J. Biol. Chem.* **279**, 46113–46121
- Park, S. S., Eom, Y. W., Kim, E. H., Lee, J. H., Min, D. S., Kim, S. J. and Choi, K. S. (2004) Involvement of c-Src kinase in the regulation of TGF- β 1-induced apoptosis. *Oncogene* **23**, 6262–6281
- Pfeffer, L. M., Yang, C. H., Murti, A., McCormack, S. A., Viar, M. J., Ray, R. M. and Johnson, L. R. (2001) Polyamine depletion induces rapid NF- κ B activation in IEC-6 cells. *J. Biol. Chem.* **276**, 45909–45913
- Pfeffer, L. M., Yang, C. H., Pfeffer, S. R., Murti, A., McCormack, S. A. and Johnson, L. R. (2000) Inhibition of ornithine decarboxylase induces STAT3 tyrosine phosphorylation and DNA binding in IEC-6 cells. *Am. J. Physiol. Cell Physiol.* **287**, C331–C335
- Daub, H., Weiss, F. U., Wallasch, C. and Ullrich, A. (1996) Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature (London)* **379**, 557–560
- Schlaepfer, D. D., Jones, K. C. and Hunter, T. (1998) Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol. Cell. Biol.* **18**, 2571–2585
- Schaffner-Reckinger, E., Gouon, V., Melchior, C., Plançon, S. and Kieffer, N. (1998) Distinct involvement of beta3 integrin cytoplasmic domain tyrosine residues 747 and 759 in integrin-mediated cytoskeletal assembly and phosphotyrosine signaling. *J. Biol. Chem.* **273**, 12623–12632
- Frisch, S. M. and Ruoslahti, E. (1997) Integrins and anoikis. *Curr. Opin. Cell Biol.* **9**, 701–706
- Meredith, Jr, J. E., Fazeli, B. and Schwartz, M. A. (1993) The extracellular matrix as a cell survival factor. *Mol. Biol. Cell* **4**, 953–961
- Zhang, Z., Vuori, K., Reed, J. C. and Ruoslahti, E. (1995) The α 5 β 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6161–6165
- Matter, M. L. and Ruoslahti, E. (2001) A signaling pathway from the α 5 β 1 and α v β 3 integrins that elevates bcl-2 transcription. *J. Biol. Chem.* **276**, 27757–27763
- Aguzzi, M. S., Giampietri, C., Marchis, F. D., Padula, F., Gaeta, R., Gianluca, R., Capogrossi, M. C. and Facchiano, A. (2004) RGDS peptide induces caspase 8 and caspase 9 activation in human endothelial cells. *Blood* **103**, 4180–4187
- Adderley, S. R. and Fitzgerald, D. J. (2000) Glycoprotein IIb/IIIa antagonists induce apoptosis in rat cardiomyocytes by caspase-3 activation. *J. Biol. Chem.* **275**, 5760–5766
- Thomas, S. M. and Brugge, J. S. (1997) Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* **13**, 513–609

-
- 43 Moro, L., Dolce, L., Cabodi, S., Bergatto, E., Erba, E. B., Smeriglio, M., Turco, E., Retta, S. F., Giuffrida, M. G., Venturino, M. et al. (2002) Integrin-induced epidermal growth factor (EGF) receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. *J. Biol. Chem.* **277**, 9405–9414
- 44 Seiler, N. (2005) Pharmacological aspects of cytotoxic polyamine analogs and derivatives for cancer therapy. *Pharmacol. Ther.* **107**, 99–119
- 45 Ribeiro, J. M. and Carson, D. A. (1993) $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease from human spleen: purification, properties, and role in apoptosis. *Biochemistry* **32**, 9129–9136
- 46 Marquet, R. and Houssier, C. (1988) Different binding modes of spermine to A-T and G-C base pairs modulate the bending and stiffening of the DNA double helix. *Biochem. Pharmacol.* **317**, 1857–1858
- 47 Wang, J. Y., Viar, M. J., McCormack, S. A. and Johnson, L. R. (1992) Effect of putrescine on S-adenosylmethionine decarboxylase in a small intestinal crypt cell line. *Am. J. Physiol.* **263**, G494–G501
- 48 Song, L., Turkson, J., Karras, J. G., Jove, R. and Haura, E. B. (2003) Activation of Stat3 by receptor tyrosine kinases and cytokines regulates survival in human non-small cell carcinoma cells. *Oncogene* **22**, 4150–4165

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