Growth factor-dependent regulation of survivin by c-myc in human breast cancer

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Abstract

Survivin has emerged as a unique regulator of cell death through its response to growth factors, such as basic fibroblast growth factor (bFGF), which we have previously shown to be mitogen-activated protein kinase (MAPK) dependent. The transcriptional complex myc/max is an oncogene that lies downstream of the MAPK pathway, suggesting a possible role in survivin's regulation. In this study, we investigated the ability of bFGF to induce signalling of the MAPK effector transcription factor c-myc in human breast cancer. Treatment of SK-BR-3 breast cancer cell line with growth factor induced survivin expression and recruitment of c-myc to its response element in the promoter region of the target gene *survivin* as demonstrated by electromobility shift analysis and chromatin immunoprecipitation assays. The promoter region of *survivin* was assessed using bioinformatic techniques and DNA footprinting. Overexpression of c-myc increased survivin protein expression. This effect was eliminated when siRNA against c-myc was transfected into the cells. c-Myc drove transcriptional activity of *survivin* when transfected into SK-BR-3 cells with a luciferase reporter vector harbouring the c-myc response element specific for *survivin*. Using confocal fluorescent microscopy, myc was located to the nucleus of breast tumour epithelial cells and was found to be significantly associated with *survivin* ($P < 0.0001$). These data provide evidence that growth factors can signal through the transcription factor c-myc in human breast cancer. They also indicate a role for c-myc in the transcriptional regulation of *survivin* in breast cancer.

Journal of Molecular Endocrinology (2006) **37**, 377–390

Introduction

Survivin is a novel member of the inhibitor of apoptosis (IAP) family of proteins (Ambrosini *et al.* 1997, Altieri 2003, Li 2003). Present during foetal development (Adida *et al.* 1998a), survivin is undetectable in most healthy, differentiated adult tissues but it is prominently expressed in transformed cell lines and most common human cancers (Ambrosini *et al.* 1997, Adida *et al.* 1998b, Chiou *et al.* 2003). Present studies show that the expression of survivin in cancer cells is associated with chemoresistance, poor prognosis, reduced disease survival and decreased apoptosis leading to cancer progression (Li 2003). Data from an *in vivo* tumour model authenticates a mechanism whereby its targeted disruption specifically abolishes tumour cells by a proapoptotic mechanism (Mesri *et al.* 2001). In addition to its ability to suppress apoptotic cell death by specifically blocking the downstream effectors of cell death (Li *et al.* 1998), survivin also plays a role in cytokinesis (Li *et al.* 1999). Consistent with the bi-functionality of survivin, there are two pools of survivin, cytoplasmic and nuclear, which are associated with evident subcellular components (Fortugno *et al.* 2002). In cancer, survivin becomes dramatically

overexpressed in response to oncogene activation and the loss of p53 (Beltrami *et al.* 2004). Survivin expression is cell cycle-regulated, with the highest levels reported in the G₂/M phase and lower levels in the G₁ phase (Li *et al.* 1998, Kobayashi *et al.* 1999). This cell cycle specificity has been credited to two cell cycle-dependent elements (CDEs) that are upstream of the transcriptional start site (Li & Altieri 1999, Otaki *et al.* 2000). Mutation within the CDEs eliminates the cell cycle specificity of survivin expression. Induction of survivin during the G₂/M phase is primarily transcriptionally regulated (Li *et al.* 1998), whereas its down-regulation on entry into G₁ is both transcriptional and post-translational; the latter mediated by the ubiquitin-proteasome pathway (Zhao *et al.* 2000). Cyclin B1/p34^{cdc} phosphorylates survivin at Thr³⁴; a modification required for survivin's anti-apoptotic activity (O'Connor *et al.* 2000a,b).

The mitogen-activated protein kinase (MAPK) pathway is a protein kinase cascade initiated by binding of growth factors to their tyrosine kinase receptors, which eventually culminate in phosphorylation and activation of downstream targets, including transcription factors, such as the proto-oncogenic transcription factor c-myc, as well as c-Fos and c-Jun. Mek phosphorylates these transcription factors, which can then

translocate to the nucleus where they may activate or suppress the transcription of target genes. We have previously shown that bFGF induces a rapid phosphorylation of c-raf in breast cancer cells and furthermore that bFGF-induced up-regulation of survivin protein can be inhibited by the MAPK/ERK Kinase (MEK) inhibitor PD98059, revealing that bFGF regulates survivin expression in an extracellular signal-regulated Kinase (ERK)1/2-dependent manner through the phosphorylation of c-raf, independently of protein Kinase B (AKT) (Teh *et al.* 2004).

c-Myc belongs to the myc family of proto-oncogenes and to the basic/helix-loop-helix/leucine-zipper (bHLHzip) class of transcription factors. It is an early-response gene that is rapidly induced by a wide range of mitogens via the MAPK pathway, and is down-regulated during differentiation (Oster *et al.* 2002). The *c-myc* gene is overexpressed in most cancers, including breast (Escot *et al.* 1986). The *c-myc* gene encodes a nuclear transcription factor with a C-terminal bHLHzip domain and an N-terminal transactivation domain. In order to activate transcription, c-myc is required to heterodimerise with another bHLH protein, Max (Blackwood & Eisenman 1991, Blackwood *et al.* 1992). The HLHzip motif allows dimerisation with the bHLHzip-protein max, which is a prerequisite for specific binding to DNA at E-box sequences (5'-CA(C/T)G(T/C)G-3') in the vicinity of target gene promoters (Blackwell *et al.* 1990, 1993, Grandori & Eisenman 1997). The proto-oncogene c-myc is at the centre of a transcription factor network that regulates cellular proliferation, replicative potential, growth, differentiation and apoptosis (Walhout *et al.* 1997, Menssen & Hermeking 2002, Oster *et al.* 2002).

Inhibitors of apoptosis aberrantly prolong cell viability and may contribute to cancer by facilitating mutations and promoting resistance to therapy. In breast cancer, growth factors including bFGF have been associated with poor prognosis and a more angiogenic phenotype. Survivin, along with other members of the *IAP* gene family, has emerged as a unique regulator of cell death through its response to locally produced growth factors, such as bFGF, which we have previously shown activate the MAPK pathway leading to the up-regulation of genes associated with tumour progression, including survivin (Teh *et al.* 2004). Survivin appears to be an important cancer therapeutic target, and modulation of survivin expression and/or function may provide rational approaches for cancer therapeutics. One of the major strategies for modulating survivin expression is control of its transcription; however, little is known regarding the molecular requirements of the basal transcriptional machinery controlling *survivin* gene expression.

The transcriptional complex myc-max is an oncogene that lies downstream of the MAPK pathway.

The development of resistance to endocrine therapy is due at least in part to a shift in the phenotype of the tumour cell from steroid dependence to that of growth factor dependence. The growth factor pathways become more prominent in endocrine resistance (Johnston *et al.* 2003). Understanding these growth factor pathways, their mechanisms and the role they play in endocrine resistance will permit the optimal integration of new signal transduction inhibitors into breast cancer therapy.

In this report, we hypothesise that bFGF can mediate survivin expression by signalling through the myc-max transcriptional network. We localised a response element for the myc-max transcriptional complex within the survivin promoter using MatInspector algorithms. Here, we report that c-myc transcriptionally up-regulates survivin by engaging its response element in the survivin core promoter region.

Materials and methods

Cell culture and reagents

The human oestrogen receptor (ER)-negative breast cancer cell line, SK-BR-3 was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Roswell Park Memorial Institute 1640 (Gibco) supplemented with 5% foetal calf serum, 50 units/ml penicillin-streptomycin and 5 µg/ml fungizone (Gibco) in a humid atmosphere incubator with 5% CO₂ at 37 °C. The cells were routinely subcultured weekly twice. The cells were incubated for 30 min, 2, 4, 6, 8, 10 or 24 h in the presence or absence of increasing concentrations of bFGF (1 and 5 ng/ml; Sigma). Where indicated, cells were pre-incubated with cycloheximide (10 µg/ml; Sigma) 60 min prior to and during addition of bFGF. The cells were removed and stored for protein or RNA extraction.

Protein extraction and quantification

Total protein (whole cell lysates) was extracted from SK-BR-3 cells by incubation in lysis buffer (PBS, 1% Igepal and 0.5% deoxycholic acid) supplemented with phenylmethylsulphonyl fluoride 10 µg/ml and aprotinin 1.5 µg/ml for 30 min. Protein was quantified using BCA assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Cytoplasmic and nuclear protein extractions of SK-BR-3 cells were performed using NE-Per nuclear and cytoplasmic extraction kits (Pierce Biotechnology, Inc.) according to the manufacturer's instructions.

Western analysis

Samples containing 50–120 µg protein were resolved on SDS-polyacrylamide gels (12% for c-myc and 15% for survivin) at 110 V for 180 min and were transferred to a nitrocellulose membrane (Bio-Rad) at 250 mA for 60 min. Membranes were incubated for 60 min in blocking buffer (5% non-fat dry milk, 0.1% Tween in TBS) at room temperature and subsequently with primary antibody, rabbit anti-human c-myc (2 µg/ml, Santa Cruz Biotechnology, CA, USA), mouse anti-human survivin (2 µg/ml, Santa Cruz Biotechnology) or rabbit anti-human phospho-c-myc (1:1000 dilution, Cell Signalling Technology, Danvers, MA, USA) in blocking buffer overnight at 4 °C. The membranes were washed before incubation with the corresponding horseradish peroxidase-conjugated mouse or rabbit secondary antibodies (1:2000 dilution; Sigma) in blocking buffer for 60 min at room temperature. The membranes were washed and developed with either Luminol (Santa Cruz Biotechnology) or enhanced chemiluminescence, SuperSignal (Pierce).

Northern analysis

Total RNA was isolated from stimulated SK-BR-3 cells using RNeasy extraction kit (Qiagen) at specific times after treatment. RNA was quantified by u.v. absorption. Ten micrograms of RNA were electrophoresed on a standard Northern gel and transferred to a nylon membrane (Bio-Rad) by pressure blotting overnight and incubation for 3 h at 80 °C. Pre-hybridisation was carried out at 42 °C for 3 h in a final buffer composition of 45% formamide, 4×SSC, 0.1 M sodium phosphate, 0.1% sodium pyrophosphate, 0.1% SDS and 250 µg/ml herring sperm. Hybridisations were performed in the same buffer at 42 °C overnight with PCR product cDNA for survivin (forward: 5'-GCA TGG GTG CCC CGA CGT TG-3'; reverse: 5'-GCT CCG GCC AGA GGC CTC AA-3') using [α -³²P]dCTP and a random primer labelling system (Promega). The blots were then washed in 2×SSC containing 0.5% SDS at room temperature for 10 min and again at 42 °C, 55 °C for 10 min before they were exposed to film at –80 °C using intensifying screens. Results were assessed directly by Eagle Eye (Stratgene, La Jolla, CA, USA) analysis and compared with levels of 28S RNA in the same lane.

Amplification of survivin promoter for use in DNase footprinting

cDNA was synthesised from 1 mg total RNA, using 50 mM oligo (dT)12–18 primers (Promega), 0.4 mM dNTPs (Promega), 1×MMLV buffer and 100 U of Moloney murine leukaemia virus (MMLV) reverse transcriptase (Invitrogen Life Technologies). PCR was

performed using primers that amplify survivin (forward: 5'-GCA TGG GTG CCC CGA CGT TG-3'; reverse: 5'-GCT CCG GCC AGA GGC CTC AA-3'). glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control (forward: 5'-GCC TCA AGA TCA TCA GCA A-3'; reverse: 5'-CCA GCG TCA AAG GTG GAG-3'). Briefly, the 25 µl reaction mix contained 1 µl cDNA, 250 µM dNTPs, 50 ng of each primer, *Taq* polymerase buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100 (TBST)) and 1.25 U *Taq* polymerase (Promega). Amplification conditions were as follows: 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 62 °C and 1 min at 72 °C with a final extension of 5 min at 72 °C. The PCR product was visualised on a 2% agarose gel. The survivin PCR product was gel purified using a DNA extraction kit (Qiagen) and sequenced (MWG, Ebersberg, Germany) to verify its identity.

Bioinformatic assessment and *in vitro* DNase I footprinting of survivin promoter

Sequence analysis of the 5' flanking region of the *survivin* gene (accession number NM_001168) using UCSC Genome Browser and Genomatrix PromoterInspector identified the putative survivin promoter. The response element for myc/max was identified within this survivin promoter using Genomatrix MatInspector.

Nuclear extracts were prepared from bFGF-treated SK-BR-3 cells as described above. Probes were generated by PCR amplification of the survivin promoter, as described in above section; the forward primer was 5'-end-labelled with [γ -³²P]ATP using polynucleotide kinase prior to use in PCR. The survivin promoter, identified through bioinformatic techniques above, was obtained using upstream primer, 5'-CTG CAC GCG TTC TTT GA-3' and downstream primer, 5'-GCG GTG GTC CTT GAG A-3'. The resulting 264 bp fragment was isolated by agarose gel electrophoresis and gel extraction (Qiagen). DNA probe (0.4 pmol) was incubated with 50–200 µg nuclear extract from bFGF-treated SK-BR-3 cells or 750 ng recombinant myc-max protein (Walhout *et al.* 1997) (kindly provided by Prof. Timmers, Department of Physiological Chemistry, University Medical Centre, Utrecht, The Netherlands) in 25 µl total volume containing 1 µg poly(dI-dC) and 20% Ficoll 400 for 30 min on ice. Following the addition of 50 µl of 5 mM CaCl₂/10 mM MgCl₂ to the samples, they were incubated for 1 min at room temperature and then submitted to DNase I cleavage (2U, Promega) by incubation at room temperature for 1 min. After phenol extraction, the DNA was ethanol precipitated and loaded in formamide dye mix (10 ml formamide, 10 mg xylene cyanol FF, 10 mg bromophenol blue) on a 6% polyacrylamide sequencing gel.

The autoradiographic banding patterns were compared with the sequenced survivin promoter using Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kits (Amersham Biosciences) according to the manufacturers' instructions.

Electrophoretic mobility shift assays (EMSA)

Nuclear protein was extracted using a NE-Per kit according to the manufacturer's instructions (Pierce). For EMSA, 1 µg nuclear extract was incubated for 30 min in the presence of 20 mM HEPES (pH 7.9), 5 mM MgCl₂, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 8% Ficoll, 600 mM KCl, 500 ng/µl poly(dI-dC) (deoxyinosinic-deoxycytidylic) acid, 50 mM DTT and [α -³²P]dCTP-labelled double-stranded oligonucleotide for c-myc response element 5'-ATG ACC ACG GGC AGA GC CACGCG GCG GGA GGA C-3' or [α -³²P]dCTP-labelled double-stranded oligonucleotide for the mutated c-myc response element 5'-ATG ACC ACG GGC AGA GC TTCCAT GCG GGA GGA C-3'. For supershift experiments, antibodies against phospho-c-myc (Santa Cruz Biotechnology) were added following the initial incubation and samples were then incubated for a further 20 min. The samples were electrophoresed through a 5.5% non-denaturing polyacrylamide gel in 0.5×Tris-borate-EDTA buffer. For competition studies, the reaction was performed as described with 100× molar excess of unlabelled probe. Supershift negative controls were performed using matched IgG control (DAKO, Glostrup, Denmark). The gel was transferred to filter paper and gel dried before being exposed to film at -80 °C using intensifying screens.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed using a ChIP assay kit (Upstate USA, Inc., Charlottesville, VA, USA) on cells that were either treated with or without bFGF for 4 h. The proteins bound to DNA were cross-linked using formaldehyde at a final concentration of 1% for 10 min at 37 °C. Then protein-DNA complexes were immunoprecipitated using 4 µg primary antibodies for c-myc (N-262, Santa Cruz Biotechnology) and anti-acetyl-histone H4 (Upstate USA, Inc.). Two 'controls' were performed – one incubated without antibody and the other with non-immune IgG. After the reversal of the cross-links by heating to 65 °C for 4 h, the DNA was recovered by phenol/chloroform extraction and precipitated by ethanol. Then the association of c-myc and H4 with the survivin promoter was measured by PCR. PCR was performed using primers that amplify survivin promoter (forward primer: 5'-CTG CAC GCG TTC TTT GA-3'; reverse primer: 5'-GCG GTG GTC CTT GAG A-3').

A pair of primers (forward primer: 5'-GCA TGG GTG CCC CGA CGT TG-3'; reverse primer: 5'-GCT CCG GCC AGA GGC CTC AA-3) selected from downstream sequence of *survivin* gene (accession number NM_001168) served as a control. Amplification conditions were as follows: 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 58 °C and 1 min at 72 °C with a final extension of 5 min at 72 °C. The samples were run on a 1.5% agarose gel and visualised by ethidium bromide staining. Parallel ChIP analyses were performed using an antiacetylated histone antibody as a positive control.

Plasmids, transient transfections and luciferase assays

Overexpression studies for c-myc were done using the pSVLneo-c-myc plasmid (kind gift from Prof. Prochownik, Children's Hospital of Pittsburgh, Pittsburgh, PA, USA). SK-BR-3 cells were transfected using a Nucleofector (Amaxa Biosystems, Cologne, Germany). The cells were transfected with 2 µg pSVLneo-c-myc or empty vector, pSVLneo, in nucleofector solution V using programme A-23. Immediately following transfection, RPMI 1640 with 5% foetal calf serum was added to the SK-BR-3 cells, which were then plated in 6-well tissue culture plates overnight. The cells were treated with and without bFGF for 4 h the following day and then collected and analysed for protein expression by Western blotting. Double-stranded oligonucleotides containing the c-myc response element (c-myc RE) specific for the survivin promoter (5'-GAC CAC GGG CAG AGC CACGCG GCG GGA GGA CTA CAA C-3') or the mutated c-myc response element (mut-c-mycRE) specific to the survivin promoter (5'-GAC CAC GGG CAG AGC TTCCAT GCG GGA GGA CTA CAA C-3'), response element for c-myc underlined, were obtained from Sigma Genosys with 5'-*Xba*I and 3'-*Bgl*II overhangs. After digesting the pGL3-Promoter, luciferase reporter vector (Promega) with *Bgl*II and *Nhe*I, the oligos were cloned into the pGL3-Promoter vector at the *Bgl*II and *Nhe*I sites in the correct orientation upstream of the luciferase reporter gene, as *Xba*I and *Nhe*I have compatible ends. The pGL3-Promoter vector contains an SV40 promoter upstream of the luciferase gene. The obtained clones, pGL3-c-mycRE and pGL3-mut-c-mycRE, were characterised by *Nhe*I and *Xba*I digestion and then confirmed by sequencing (MWG). SK-BR-3 cells were transfected using a Nucleofector as described above. Each of the relevant c-myc survivin-specific promoter-luciferase constructs were co-transfected with pRL-TK (TK promoter-Renilla-luciferase construct, internal control) with or without an expression plasmid for c-myc, pSVLneo-c-myc or the Silencer c-myc-validated siRNA (Cat no. 4250, Ambion, Austin, TX, USA). The cells were processed for luciferase assays

24 h post-transfection. For the luciferase assay, a dual-luciferase reporter assay system (Promega) was used. The transfected cells in 6-well plates were washed with PBS and lysed with 1× passive lysis buffer (up to 500 µl/well) on a shaker for 15 min at room temperature. Cell lysate (20 µl/well) was used for the measurement of luciferase activities in a luminometer by first mixing the cell lysate (20 µl) with 100 µl luciferase assay reagent for measuring firefly luciferase activity and subsequently adding 100 µl Stop-Glo reagent for measuring Renilla luciferase activity. Data were normalised to Renilla luciferase activity (internal control) as arbitrary units.

siRNA transfection

Validated siRNAs by Ambion were used. The Silencer c-myc-validated siRNA (Cat no. 4250) was assessed for its ability to down-regulate c-myc protein expression. Silencer Negative Control no. 1 (scrambled) siRNA or Silencer GAPDH siRNA (Human; Ambion) was used according to the manufacturer's instructions as control siRNAs. SK-BR-3 cells were transfected using a Nucleofector (Amaxa Biosystems). The cells were transfected with 1.5 µg siRNA in Nucleofector solution V using programme A-23. Immediately following transfection, RPMI 1640 with 5% foetal calf serum was added to the SK-BR-3 cells, which were then plated in 6-well tissue culture plates overnight. The cells were collected the following day and analysed for protein expression by western blotting.

Patient population and clinical characteristics

Following ethical approval from St Vincent's University Hospital, patients were selected on the basis of those who received tamoxifen only as adjuvant therapy after surgery. All patients had stage I–II breast cancer at presentation and were assessed by abdominal ultrasound, chest X-ray and bone scintigraphy before surgery. None of the group received adjuvant chemotherapy after their initial surgery. All patients received tamoxifen 20 mg/day for 5 year, which was discontinued only in those patients who suffered a relapse, while on endocrine therapy. In those patients who were ER negative, tamoxifen was prescribed on the basis that they were progesterone receptor positive.

Immunodetection/microscopy

Immunodetection was carried out on fixed SK-BR-3 breast cancer cells and primary breast cancer tissue. Immunohistochemistry was used to detect survivin and c-myc on paraffin-embedded tissue. Sections (5 µm)

were cut from paraffin-embedded breast tumour blocks and mounted on slides. Sections were dewaxed and re-hydrated. Antigen retrieval was performed by immersing sections in 0.6 M citrate buffer and microwaving on high power for 7 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min. Antigens were detected using the Vectastain Elite kit (Vectra Labs, Burlingame, CA, USA) according to the manufacturer's instructions. Briefly, cells and sections were blocked in serum for 90 min. The cells and sections were incubated with primary antibodies, mouse anti-human survivin (4 µg/ml) and rabbit anti-human c-myc (1 µg/ml) (Santa Cruz Biotechnology) for 60 min at room temperature. Subsequently, sections were incubated in the corresponding biotin-labelled secondary antibody (1 in 2000) for 30 min, followed by peroxidase-labelled avidin–biotin complex. Sections were developed in 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and counterstained with haematoxylin (Sigma). Negative controls were performed using matched IgG controls (DAKO). Sections were examined under a light microscope. Immunostained slides were scored using the Allred scoring system (Harvey *et al.* 1999). First, a proportion score was assigned, which represented the estimated portion of positively stained tumour cells (none=0; <1%=1; >1% and <10%=2; >10% and <33%=3; >33% and <66%=4; >66%=5). Next, an intensity score was assigned that represented the average intensity of the positive tumour cells (none=0; weak=1; intermediate=2; strong=3). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8. A total score greater than 2 was taken to indicate positivity. Independent observers, without the knowledge of prognostic factors, scored slides.

Immunofluorescence detection of survivin and c-myc was performed on SK-BR-3 cells and primary breast cancer tissue. The cells and tissues were prepared as described above. SK-BR-3 cells were cultured on 8-well chamber slides (Lab Tek, Campbell, CA, USA) and subsequently fixed and permeabilised. Breast cancer cells and sections were blocked in 1.5% normal serum and then incubated with rabbit anti-human c-myc (10 µg/ml in 10% human serum) for 90 min. The sections were incubated with the corresponding secondary Alexa Fluor 594 goat anti-rabbit antibody (1 in 200) (Molecular Probes, Paisley, UK) for 60 min. Subsequently, the slides were blocked in sheep serum for 90 min. Each slide was incubated with mouse anti-human survivin (40 µg/ml in 10% human serum) for 90 min. The slides were incubated with the corresponding secondary Oregon Green 488 goat anti-mouse antibody (1 in 200) (Molecular Probes) for 60 min. All steps were preceded by a wash with PBS. The sections were counterstained with DAPI (Sigma-Aldrich) (1 in 1000 in dH₂O). Negative controls were

performed using matched IgG (DAKO) and omission of the primary antibody. The sections were mounted using fluorescent mounting media (DAKO). The slides were examined under a fluorescent microscope. Confocal microscopy was performed using a confocal microscope (Zeiss LSM 510 UV META system) and images were captured using Laser Capture software.

Clinicopathological parameters

Variables analysed included: patient age, tumour size, tumour grade, human epidermal growth factor receptor-2 (HER2) status and axillary node status. A recurrence was defined as any local (chest wall) or systemic (visceral or bone metastasis) recurrence during the follow-up period.

Statistical analysis

Statistical analysis was carried out using the χ^2 -test for categorical variables. Two-sided *P* values of <0.05 were considered to be statistically significant. Statistical analysis was performed using SPSS statistical software (version 14, Chicago, IL, USA).

Results

Growth factor regulation of survivin expression

In previous studies, we have shown that bFGF induces a rapid phosphorylation of c-raf in breast cancer cells and furthermore that bFGF-induced up-regulation of survivin protein can be inhibited by the MAP kinase antagonist PD98059, suggesting that bFGF regulates

survivin expression in an ERK1/2-dependent manner through the phosphorylation of c-raf, independently of AKT (Teh *et al.* 2004). To further evaluate the early and the late regulation of survivin following stimulation with bFGF in breast cancer cells, we have investigated the ability bFGF to modulate survivin protein and mRNA expression in the human breast cancer cell line, SK-BR-3. The SK-BR-3 breast cancer cell line was chosen for use in this study because of the high endogenous levels of survivin expression. bFGF (1–5 ng/ml) induced survivin protein expression, in a dose-dependent manner, following 30-min incubation and survivin continued to be up-regulated following 24 h of treatment (Fig. 1a). To assess if the latent regulation of survivin expression requires *de novo* protein synthesis, SK-BR-3 cells were pre-treated with cycloheximide. Inhibition of *de novo* protein synthesis with cycloheximide abrogated bFGF-induced up-regulation of survivin protein from 2 h, but not at the earlier time point of 30 min (Fig. 1a). There was no increase in survivin mRNA expression at 30 min in the presence of bFGF; however, when the breast cancer cells were treated with bFGF for 6 h, a marked increase in the expression of mRNA was detected (Fig. 1b). These observations suggest it is only the later regulation of survivin that is under transcriptional control.

Defining a transcriptional response element in the survivin promoter

Little is known regarding the molecular requirements of the transcriptional machinery controlling *survivin* gene expression. In order to identify what transcription factor is involved in the transcriptional regulation of survivin, we assessed the DNA upstream of the *survivin*

Figure 1 bFGF up-regulates survivin protein and mRNA expression. (a) Western blot analysis of survivin in SK-BR-3 breast cancer cells pre-treated with or without cycloheximide (CHX) (10 μ g/ml) for an hour prior to and during incubation with bFGF as indicated. Results are representative of those obtained in three separate experiments. (b) Survivin mRNA expression was determined by Northern blotting following incubation with bFGF as indicated. The presence and integrity of the 28S major RNA component for each lane was examined and found to be similar.

Figure 2 DNase footprinting and EMSA revealed that c-myc binds its response element in the *survivin* promoter. (a) DNA sequence analysis of the 5' flanking region of the human *survivin* gene through Genomatrix PromoterInspector revealed a putative *survivin* promoter (bold) as shown. Numbering is from the initiating ATG. The response element for *myc/max* identified within the promoter of *survivin* by Genomatrix MatInspector is underlined, with base pairs in capital letters denoting the core sequence. The forward and reverse primers used to amplify the core 264 bp *survivin* promoter used in DNase footprinting reactions are indicated with horizontal arrows. First, exon 1 is in higher case. (b) DNase footprinting identified c-myc binding to its response element in the *survivin* promoter. Nuclear protein extracts from bFGF-treated SK-BR-3 cells were incubated with 5'-end-labelled [γ - 32 P]ATP *survivin* promoter and analysed for protected footprints. The autoradiographic banding patterns were compared with the sequenced *survivin* promoter using Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit (Amersham Biosciences) according to the manufacturers' instructions. (c) Nuclear protein extracts from SK-BR-3 cells in the presence and the absence of 5 ng/ml bFGF were compared for increased binding to α - 32 P-dCTP-labelled c-myc response element (upper panel) and α - 32 P-dCTP-labelled mutated c-myc response element (lower panel). DNA protein interactions were assayed in the presence of 100 $\times$ molar excess of homologous oligonucleotide. Nuclear protein extracts were pre-incubated in the presence of anti-phospho-c-myc. (d) Western blot analysis of c-myc and phospho-c-myc in SK-BR-3 breast cancer cells treated with bFGF as indicated. Results are representative of those obtained in three separate experiments.

gene. Using bioinformatic techniques (UCSC Genome Browser, PromoterInspector), analysis of the DNA sequence of the 5' flanking region of the human *survivin* gene revealed a putative survivin promoter (bold) as shown in Fig. 2a. The probable promoter region was identified upstream of the initiating ATG and including part of the first exon (Fig. 2a). Further bioinformatical assessment of the survivin promoter, using MatInspector, uncovered a putative myc-max response element, gcCACGCGgc, with base pairs in capital letters denoting the core sequence (Fig. 2a). Sequence analysis of the survivin promoter, for other transcription factor response elements using MatInspector, also revealed response elements for the Sp1 and E2F family of transcription factors as previously identified by others (Li & Altieri 1999, Jiang *et al.* 2004). The putative myc-max response element identified through bioinformatics was confirmed by successful binding of breast cancer nuclear extracts and recombinant c-myc protein to the myc-max response element using DNase footprinting (Fig. 2b). To determine the ability of bFGF to induce increased transcription

factor-DNA binding at the myc-max response element, electromobility gel shift analysis was carried out. Using an oligonucleotide sequence, which is specific for the survivin promoter, the ability of nuclear extracts from non-treated SK-BR-3 breast cancer cells (Fig. 2c, upper panel) to bind to the DNA response element was compared with cells treated with bFGF. c-Myc response element binding was induced in the presence of bFGF in comparison with control. An immunodepletion induced by pre-incubation of the nuclear extracts with anti-phospho-c-myc established that the c-myc transcription factor was present at the protein-DNA complex. Binding was specific as demonstrated by both successful competition with excess homologous oligonucleotide, and no observed binding when the myc-max response element was mutated (Fig. 2c, lower panel). To determine if mitogen stimulation induces c-myc expression, we treated SK-BR-3 cells with bFGF (1–5 ng/ml) and saw that bFGF induces c-myc and phospho-c-myc at the early time point of 30 min (Fig. 2d). No alteration of c-myc or phospho-c-myc protein expression was seen at the later time point of

Figure 3 Recruitment of c-myc to the survivin promoter in response to growth factors can regulate survivin promoter activity (a) SK-BR-3 cells were treated with bFGF (5 ng/ml) for 4 h. The cells were then subjected to the ChIP protocol. The immunoprecipitations were performed with antibodies against c-myc (lanes 8 and 9), acetylated histone H4 (lanes 6 and 7) or no antibody (– Ab, lane 5) and non-immune IgG (lane 4) as negative controls. A set of primers amplifying a distal downstream region of the *survivin* gene were also used as a control for PCR (lane 3). Human genomic DNA (Promega) was used as a positive control (lane 1). The isolated promoter DNA fragments were amplified using a pair of primers covering the c-myc response element of the *survivin* gene promoter. Inputs represent soluble chromatin that was reversed cross-linked and amplified by PCR (30 cycles). (b) and (c) SK-BR-3 cells were transfected with the pGL3 plasmid containing the c-myc response element (c-myc RE) specific for the survivin promoter, pGL3-c-mycRE or the mutated c-myc response element (mut-c-mycRE) specific to the survivin promoter, pGL3-mut-c-mycRE, and the internal control vector, pRL-TK. The effect of knocking-down c-myc on the survivin promoter-luciferase construct was observed when the cells were co-transfected with c-myc siRNA. The cells were treated with bFGF for 6 h as indicated, followed by measurement of luciferase activity using a dual luciferase reporter system as described under 'Materials and methods'. Luciferase activity was normalised to *Renilla* luciferase and shown in arbitrary units in a histogram. Each bar is the mean $\pm$ s.d. from a representative experiment carried out in triplicate.

4 h confirming c-myc status as an immediate early gene (Fig. 2d).

c-Myc transcriptional activation of survivin expression

To investigate if c-myc can be recruited to the survivin promoter in breast cancer cells, ChIP assays were carried out (Fig. 3a). SK-BR-3 cells were exposed to bFGF (5 ng/ml) for 4 h, and chromatin was isolated following chemical cross-linking. The cross-linked chromatin was fragmented and immunoprecipitated with antibodies against c-myc and acetylated histone H4. The DNA obtained from immunoprecipitated chromatin was amplified using primers flanking the E-box site in the survivin promoter (Fig. 2a). Our results indicated that binding was not detectable under control conditions (Fig. 3a, upper panel, lane 8). However, in the presence of bFGF, c-myc exhibited binding to the survivin promoter (Fig. 3a, upper panel, lane 9). Consistent with gene activation following stimulation with bFGF, acetylated histone H4 was detected at the survivin promoter (Fig. 3a, upper panel, lanes 6 and 7). These results indicated that growth factor-mediated expression of *survivin* gene involves interaction of c-myc with the E-box region within the survivin promoter.

To further elucidate the ability of c-myc to transcriptionally activate survivin expression, we employed luciferase reporter assay experiments. An oligo, specific for the survivin promoter and containing the myc-max response element, was cloned into the pGL3-promoter vector (pGL3-c-mycRE) to assess a transcriptional mechanism for the modulation of survivin by the transcription factor c-myc. Exogenous c-myc increased the enhancer activity of an isolated myc response

element specific for the survivin promoter (Fig. 3b) and this expression was suppressed when siRNA specific for c-myc was co-transfected with the reporter and the expression vectors (Fig. 3b). No increase in the enhancer activity of an isolated myc response element specific for the survivin promoter was observed when the oligonucleotide specific for survivin promoter containing the myc-max response element was mutated (pGL3-mut-c-mycRE) (Fig. 3b). To confirm the role of bFGF in the c-myc regulation of survivin expression, we used luciferase reporter assays in cells treated for 6 h with 5 ng/ml bFGF following transfection with pGL3-c-mycRE (Fig. 3c). This revealed an increase in the enhancer activity of an isolated myc response element specific for the survivin promoter, which was suppressed when siRNA specific for c-myc was co-transfected with the reporter and the expression vectors, indicating that c-myc is central to this mechanism of regulation.

The effect of c-myc on survivin expression

c-Myc functions as a transcriptional activator downstream of the MAPK signalling pathway through heterodimerisation with its partner max. Here, c-myc expression was rapidly induced by the growth factor bFGF (Fig. 4a, right-hand panel). To determine if c-myc can control survivin expression, c-myc was transiently transfected into SK-BR-3 breast cancer cells and a corresponding increase in survivin protein expression was observed (Fig. 4a, left-hand panel). Up-regulation of c-myc and survivin protein expression by bFGF was observed in cells transfected with the pSVLneo backbone of the c-myc overexpressing vector, pSVLneo-c-myc (Fig. 4a, right-hand panel). No further increase

Figure 4 The effect of c-myc expression on survivin. (a) SK-BR-3 cells were transfected with the overexpression vector for c-myc, pSVLneo-c-myc with its backbone vector, pSVLneo. Twenty-four hour post-transfection protein expression of both survivin and c-myc was determined by Western blotting (left panel). The cells were also treated with bFGF for 4 h as indicated 24-h post-transfection and the effect on c-myc and survivin protein expression was examined (right panel). (b) Scrambled siRNA and siRNA for c-myc (Ambion) were transfected into SK-BR-3 cells and the protein expression of c-myc and survivin was determined by western blotting 24-h post-transfection.

in protein expression of c-myc was observed in cells transfected with pSVLneo-c-myc when treated with bFGF, probably due to the saturation of protein in cells overexpressing c-myc. To confirm the relationship between c-myc and survivin, c-myc protein expression was knocked-down following transfection with siRNA specific for c-myc and a corresponding decrease in survivin protein expression was observed (Fig. 4b).

Localisation of c-myc and survivin expression in human breast cancer

Survivin and the transcription factor c-myc were localised within paraffin-embedded human breast tissue using immunohistochemistry. Survivin was exclusively expressed in the breast cancer epithelial cells and not in the normal surrounding tissue (Fig. 5a). Survivin was predominantly expressed in the cytosol of the tumour epithelial cells with some nuclear staining also detected. c-Myc was found primarily in the nuclei of breast epithelial cells; however, scant staining was also detected within the cytosol (Fig. 5a). In order for c-myc to have a role in the transcriptional regulation of survivin, the IAP family member and the transcription factor must both be expressed within the same cell.

The ability of breast epithelial cells to co-express both survivin and c-myc was successfully demonstrated using immunofluorescence and confocal microscopy (Fig. 5b).

To assess the potential clinical relevance of the association between survivin and the transcription factor c-myc *in vivo*, expression of survivin and c-myc was analysed by immunohistochemistry in a cohort of breast cancer patients ($n=70$). Qualitative expression of survivin and c-myc, both alone and in combination, were correlated with clinicopathological data (Table 1). No relationship between the expression of survivin alone, or in combination with c-myc, was observed in relation to patient age, tumour size, axillary node status, ER expression and recurrences (Table 1). Survivin was found to positively associate with histological grade ($P=0.012$) and HER2 status ($P=0.044$). A highly significant correlation between survivin and c-myc ($P<0.0001$) was observed in the cohort of primary breast tumours. Thus, the relationship between c-myc and increased survivin expression, as observed in SK-BR-3 tumour cell line through molecular techniques, was also relevant in primary breast cancers. These data taken together support a role for c-myc in survivin's regulation.

Figure 5 Localisation of survivin and c-myc in human breast cancer tissue. (a) Immunohistochemical localisation of survivin and c-myc counterstained with haematoxylin and matched IgG negative controls in human breast cancer tissue. (b) Immunofluorescent co-localisation of survivin with c-myc by confocal microscopy (magnification $\times 630$) in human breast cancer tissue (upper panel) and in SK-BR-3 breast cancer cell line (lower panel).

Table 1 Associations of survivin and c-myc, alone and in combination with clinicopathological parameters, comparisons with χ^2 -test

	Total ^a	Survivin positivity (%) ^a	P value	c-Myc positivity (%) ^a	P value	Survivin and c-myc positivity (%) ^a	P value
No. of patients	70	32 (46%)	—	42 (60%)	—	25 (36%)	—
Patient age (years)							
≤ 50	31	14 (35%)	0.799	18 (56%)	0.619	10 (32%)	0.337
> 50	34	13 (38%)		19 (56%)		10 (29%)	
Survivin expression							
Survivin positivity	32	—	—	26 (81%)	<0.0001	—	—
Survivin negativity	38	—		16 (42%)		—	
Tumour size (mm)							
≤ 35	47	21 (45%)	0.962	27 (57%)	0.535	16 (34%)	0.908
> 35	22	10 (45%)		14 (64%)		8 (36%)	
Histological grade							
Grade 3	33	10 (30%)	0.012	19 (58%)	0.702	9 (27%)	0.109
Non-grade 3	37	22 (59%)		23 (62%)		16 (43%)	
Node status							
Node positive	49	20 (41%)	0.799	27 (55%)	0.619	16 (33%)	0.337
Node negative	21	12 (57%)		15 (71%)		9 (43%)	
HER-2-neu expression							
HER-2-neu positivity	26	16 (62%)	0.044	17 (65%)	0.420	13 (50%)	0.066
HER-2-neu negativity	44	16 (36%)		25 (57%)		12 (27%)	
ER expression							
ER positivity	53	21 (40%)	0.090	31 (58%)	0.511	17 (32%)	0.297
ER negativity	17	11 (65%)		11 (65%)		8 (47%)	
Recurrences							
Recurrence	14	4 (29%)	0.219	6 (43%)	0.213	3 (21%)	0.147
Non-recurrence	47	23 (49%)		30 (64%)		20 (43%)	

^aValues and percentages are expressed from available data.

Discussion

The precise molecular mechanism of survivin transcriptional regulation remains unclear. Understanding the mechanisms controlling *survivin* gene transcription could help lead to a rationalised and targeted approach for inhibiting this protein, which has been linked with decreased apoptotic index and poor survival rates in patients with survivin-positive tumours (Tanaka *et al.* 2000). Here, we report *ex vivo* studies on human breast cancer tissue and *in vitro* functional studies on SK-BR-3 breast cancer cells. We describe a novel mechanism for survivin regulation by the transcription factor c-myc. In this study, we present molecular and translational data to support the hypothesis, that under the influence of growth factors, c-myc is a functional transcriptional regulator of the anti-apoptotic protein survivin in human breast cancer.

Survivin expression has been previously shown to be under the control of circulating growth factors and cytokines (Tran *et al.* 1999, O'Connor *et al.* 2000a,b). In previous studies, we have demonstrated that the growth factor bFGF can up-regulate survivin expression in breast cancer cells, leading to increased cell proliferation with a concomitant decrease in apoptosis (Teh *et al.* 2004). We and others have found that growth factor regulation of survivin expression is dependent

on the MAP kinase signalling pathway (Carter *et al.* 2001, Teh *et al.* 2004). Here, increases in survivin mRNA were detected at 6 h following bFGF treatment. In this study, we found bFGF up-regulation of survivin to be biphasic, with early increases in survivin protein detected at 30 min and later increases seen from 2 h following treatment. Inhibition of *de novo* protein synthesis with cycloheximide altered bFGF-induced expression from 2 h following treatment. Moreover, increases in survivin mRNA were also detected only at the later time period. Taken together, these data suggest that rapid elevation of survivin occurs at the translational or post-translational level, whereas later regulation by bFGF can occur at the level of transcription.

Survivin is aberrantly expressed in the majority of human tumours; however, the mechanism of its de-regulation in these tumours has not been determined. It is unlikely that high levels of expression occur exclusively as a result of gene amplification, as amplicons of the survivin locus have not been observed. More likely, regulation of survivin expression occurs at the level of transcription as a consequence of the de-regulation of upstream transcriptional regulators (Jiang *et al.* 2004). To date, little is known regarding the transcriptional regulation of survivin. This study reports that c-myc transcriptionally up-regulates the expression

of the anti-apoptotic protein survivin, providing a molecular mechanism for its regulation by growth factors in human breast cancer. In addition to c-myc, several other factors are found to be activators of survivin transcription, namely, β -catenin/TCF (T-cell factor) (Ma *et al.* 2005), Nuclear factor- κ B (Kawakami *et al.* 2005), E2F family members (Jiang *et al.* 2004), signal transducer and activator of transcription-3 (STAT3; Kanda *et al.* 2004) and differentially expressed in chondrocytes (DEC1; Li *et al.* 2006). Consistent with abundant expression of survivin in tumours, the activity of these factors is notably increased in a variety of cancers (Jiang *et al.* 2004, Kanda *et al.* 2004, Kawakami *et al.* 2005, Ma *et al.* 2005, Li *et al.* 2006). The increased transcription activity is achieved by overexpression, as shown by E2F1 and DEC1, post-translational modification shown by STAT3, or nuclear translocation observed with β -catenin (Li *et al.* 2006). Using deletion mutants, studies have revealed that these transcription factors activate the survivin promoter by binding their respective response elements in the proximal promoter region (Jiang *et al.* 2004, Kawakami *et al.* 2005, Ma *et al.* 2005, Li *et al.* 2006).

Expression of the IAP is cell cycle regulated, with the highest levels found in the G₂/M phase. Survivin has a TATA-less promoter containing a canonical CpG island, three cell cycle-dependent elements, one cell cycle homology region and numerous Sp1 sites (Li & Altieri 1999). The cell cycle expression of survivin is thought to be dependent on Sp1, with transcriptional activity requiring two critical Sp1 sites (Li & Altieri 1999). The two critical Sp1 sites (−151 and −171) (numbering from initiating ATG) identified by Li & Altieri (1999) for constitutive activation of survivin in HeLa cells are different to those Sp1 sites (−127 and −226) identified by Li *et al.* (2006) for DEC1 activation of survivin, suggesting cell type-specific regulation of survivin. In this study, bioinformatical assessment of the survivin proximal promoter using MatInspector identified binding regions for E2F and Sp1 as identified by others (Li & Altieri 1999, Jiang *et al.* 2004). Analysis also revealed an E-box (CACGCG), a putative binding region for the MAP kinase effector, myc-max. These data, coupled with the observation that growth factor regulation of survivin expression is MAP kinase dependent, led to the hypothesis that c-myc could function as a transcriptional regulator of survivin in breast cancer cells.

In this study, EMSA and ChIP analysis of the survivin promoter demonstrated that c-myc could be recruited to this E-box within the survivin promoter under the influence of bFGF. Furthermore, using siRNA technology and luciferase assays we observed that growth factor regulation of survivin was, at least in part, dependent on the transcription factor c-myc. The c-myc response element oligos specific for survivin

were identified initially through bioinformatical assessment of the survivin promoter and verified with DNase footprinting and EMSA. In the reporter assays using these, along with mutated c-myc response element oligos, we specifically manipulated the c-myc response element in relation to the survivin promoter and observed bFGF induction of survivin transcription. Following our previous observation that bFGF-induced regulation of survivin is MAPK dependent (Teh *et al.* 2004), we have now shown that bFGF can induce c-myc protein expression in addition to directly phosphorylating c-myc in a MAPK-dependent manner. These findings subsequently led us to deduce the following survivin activation pathway whereby bFGF activates the MAPK cascade leading to phosphorylation of the transcription factor c-myc and ultimately resulting in elevated *survivin* gene expression.

It is estimated that survivin is up-regulated in around 60–70% of breast cancers (Tanaka *et al.* 2000, Kennedy *et al.* 2003). This high frequency of expression in cancer suggests that survivin may be involved in tumourigenesis. In addition to poor response to chemotherapy, up-regulation of survivin has generally been associated with increased tumour aggressiveness and decreased patient survival rates (Adida *et al.* 1998a,b, Tamm *et al.* 1998). To determine if the above molecular observations could be relevant in an *ex vivo* setting, we assessed expression of both survivin and c-myc within human breast cancer tissue. Using an antibody raised against the full-length protein, detecting both nuclear and cytoplasmic forms, we found survivin to be expressed in 46% of breast cancer patients. Survivin was localised to the breast tumour epithelial cells. At a subcellular level, survivin was found to be predominantly cytoplasmic, though some nuclear expression was also observed. These data are consistent with previous findings that survivin exists in distinct subcellular pools, with the anti-apoptotic form being cytoplasmic and the spindle formation form nuclear (Fortugno *et al.* 2002). c-Myc's correlation with prognosis and therapy is controversial and may vary in different tissue types of malignancy (Liao & Dickson 2000, Schlotter *et al.* 2003). The c-myc transcription factor is one of the most potent and frequently deregulated oncoproteins in human cancers (Dang 1999, Prochownik 2004). c-Myc expression was detected in 60% of our patient population, which is in agreement with the levels reported by previous immunohistochemical analyses of c-myc in breast cancer, with expression varying from 50 to 100% positivity (Spaventi *et al.* 1994, Pietilainen *et al.* 1995, Sierra *et al.* 1999). c-Myc was found to be predominantly nuclear; however, localisation to the cytoplasm is not unusual, as despite being a transcription factor it is cytoplasmic in its unphosphorylated state, and temporarily translocates to the nucleus following

phosphorylation and activation by ERK 1/2. In this study of a cohort of breast cancer patients ($n=70$), expression of survivin alone positively correlated with histological grade and HER2 status. Our findings have also been observed by Chu *et al.* (2004) who found a significant relationship between survivin and HER2 overexpression in primary breast tumours. The anti-apoptotic effects of HER2 were shown to be mediated by signalling through the phosphatidylinositol-3-kinase (PI3-K)/AKT pathway (Arboleda *et al.* 2003). Indeed, signalling via PI3-K/AKT was previously shown to up-regulate survivin expression (Papapetropoulos *et al.* 2000, Carter *et al.* 2001, Fukuda & Pelus 2002). Thus, it is possible that HER2, by acting through the PI3-K/AKT pathway, may block apoptosis by up-regulating expression of survivin. A highly significant association between expression of survivin and c-myc ($P<0.0001$) is also reported here and supports the proposal that c-myc is involved in the regulation of survivin, and highlights a possible novel signalling pathway that has potential as a therapeutic target.

In this study, we present molecular and translational data to support the hypothesis that c-myc is a functional transcriptional regulator of survivin in human breast cancer. Defining c-myc as a growth factor-dependent regulator of survivin provides a functional link between these two anti-apoptotic proteins in breast tumour progression.

Funding

This work was supported by a grant from Cancer Research Ireland (CRI02YOU). The authors declare that there is no conflict of interest that would prejudice its impartiality.

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Received 3 July 2006

Accepted 12 July 2006