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ORIGINAL ARTICLE

Regulation of survivin expression by IGF-1/mTOR signaling

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Survivin is a dual regulator of cell proliferation and cell viability overexpressed in most human tumors. Although strategies to lower survivin levels have been pursued for rational cancer therapy, the molecular circuitries controlling survivin expression in tumors have not been completely elucidated. Here, we show that stimulation with insulin-like growth factor-1 (IGF-1) results in increased survivin expression in prostate cancer cells. This response is independent of *de novo* gene transcription, changes in mRNA expression or modifications of survivin protein stability. Instead, IGF-1 induced persistence and translation of a pool of survivin mRNA, in a reaction abolished by the mTOR (mammalian target of rapamycin) inhibitor, rapamycin. Forced expression of the mTOR target p70S6K1 reproduced the increase in survivin expression in prostate cancer cells, whereas acute ablation of endogenous p70S6K1 by small interfering RNA down-regulated survivin levels. Rapamycin, alone or in combination with suboptimal concentrations of taxol reduced survivin protein levels, and decreased viability of prostate cancer cells. Therefore, IGF-1/mTOR signaling elevates survivin in prostate cancer cells via rapid changes in mRNA translation. Antagonists of this pathway may be beneficial to lower an antiapoptotic threshold maintained by survivin in prostate cancer.
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Keywords: survivin; IGF-1; prostate cancer; mTOR; rapamycin

Introduction

Deregulation of apoptosis leading to aberrantly increased cell survival is a hallmark of cancer (Hanahan and Weinberg, 2000), and may result from increased expression of cell death antagonists or loss of cell death inducers (Hengartner, 2000). Restoration of apoptotic pathways is being explored for rational cancer therapy (Reed, 2003), and targeting survival proteins of the Bcl-2 (Cory and Adams, 2002), or inhibitor of apoptosis (IAP) (Salvesen and Duckett, 2002) gene family has been associated with tumor cell death, and in some cases, promising clinical responses (Reed, 2003). Survivin (Ambrosini *et al.*, 1997) has attracted attention as a unique IAP member (Salvesen and Duckett, 2002) for its differential expression in tumors as opposed to normal tissues (Ambrosini *et al.*, 1997), and a role in multiple pathways of tumor cell maintenance, including protection from apoptosis, cell division, the cellular stress response and p53-dependent checkpoints (Altieri, 2003).

Although the differential expression of survivin in tumors is influenced transcriptionally by oncogenic signaling (Altieri, 2003), and loss of p53-mediated gene repression (Hoffman *et al.*, 2002; Mirza *et al.*, 2002), other, non-transcriptional regulatory mechanisms have been identified. These include rapid changes in survivin protein stability modulated by phosphorylation (O'Connor *et al.*, 2000), subcellular trafficking controlled by monoubiquitination (Vong *et al.*, 2005) and dynamic exchange of survivin pools among individual subcellular compartments (Dohi *et al.*, 2004). In prostate cancer, a plethora of molecular pathways has been associated with modulation of survivin levels, including extracellular matrix–integrin interactions (Fornaro *et al.*, 2003), antiandrogen therapy (Zhang *et al.*, 2005), Stat3 activation (Nam *et al.*, 2005) and Smad/BMP-7 signaling (Yang *et al.*, 2006), thus contributing to apoptosis resistance. In addition, activation of PI3 kinase/Akt signaling, which is a common hallmark of prostate cancer, has been consistently linked to increased survivin levels (Dan *et al.*, 2004), but the downstream target(s) of this response have not been identified.

Here, we studied a potential link between survivin expression and the mammalian target of rapamycin (mTOR) (Wullschleger *et al.*, 2006), a downstream effector of Akt with critical roles in cell growth, cell survival and adaptation to stress (Hay, 2005).

Results

IGF-1 modulation of survivin expression

Serum-deprived prostate adenocarcinoma DU145 cells exhibited low expression of endogenous survivin, which was induced by insulin-like growth factor (IGF-1) in a

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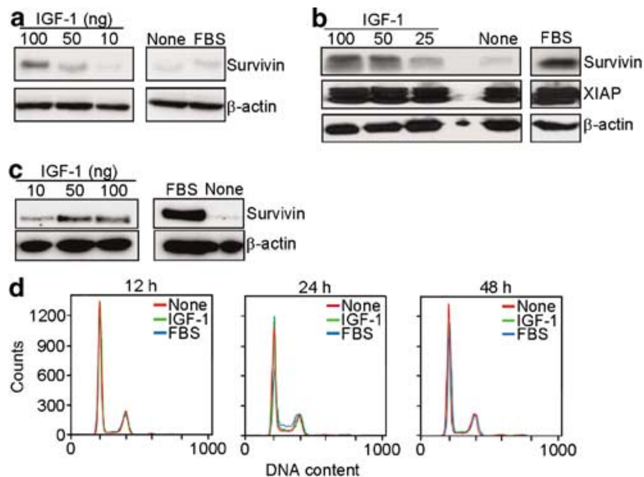

Figure 1 Regulation of survivin expression by IGF-1 stimulation. (**a–c**) Serum-deprived DU145 cells were stimulated with the indicated concentrations of IGF-1 (ng/ml) or 10% FBS, and analysed after 12 h (**a**), 24 h (**b**) or 48 h (**c**), by immunoblotting. (**d**) Cell cycle analysis. Serum-deprived DU145 cells were stimulated with medium (None), 10% FBS or IGF-1 (100 ng/ml), and analysed for DNA content by propidium iodide staining and flow cytometry at the indicated time intervals.

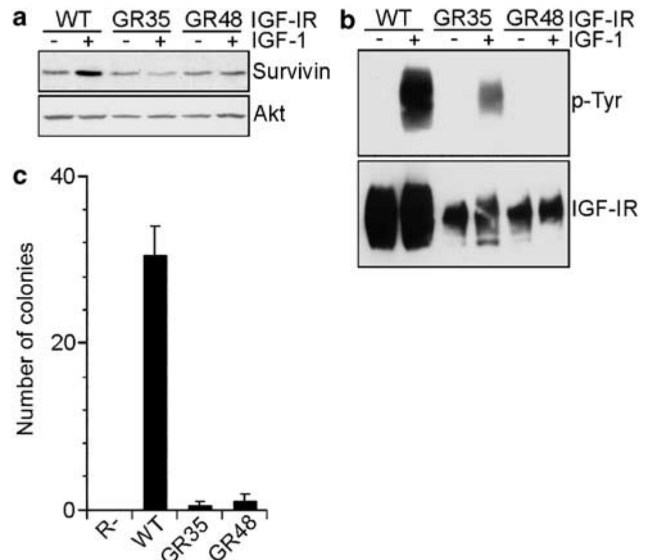

Figure 2 IGF-IR transforming potential is required for IGF-1-induced expression of survivin. (**a**) Modulation of survivin expression. MEF from IGF-IR null mice (R−) stably transfected to express WT, GR35 or GR48 mutant IGF-IR were stimulated with IGF-1, and analysed by immunoblotting. (**b**) IGF-IR tyrosine phosphorylation. Cells were stimulated with IGF-1 (100 ng/ml), and lysates were immunoprecipitated with an antibody to IGF-IR, followed by immunoblotting. p-Tyr, tyrosine-phosphorylated proteins. (**c**). Colony formation assay. The indicated transfected cells were seeded in semisolid medium, and colonies larger than 100 μm were counted after 14 days. Data are the mean ± s.e.m. of triplicates.

concentration-dependent manner, by immunoblotting (Figure 1a). IGF-1 induction of survivin was detectable as early as 12 h after stimulation (Figure 1a), and persisted throughout a 24 h (Figure 1b) or 48 h (Figure 1c) time interval. In contrast, mitogen stimulation of DU145 cells with fetal bovine serum (FBS) did not modulate survivin levels after 12 h (Figure 1a), but increased survivin expression at 24 and 48 h after treatment (Figure 1b and c). The effect of IGF-1 was specific for survivin, as FBS or IGF-1 did not modulate the expression of another IAP member, XIAP, or β-actin (Figure 1a–c). Finally, IGF-1 stimulation did not significantly affect the DNA content profile of DU145 cells at comparable time intervals (Figure 1d), suggesting that changes in survivin expression after exposure to IGF-1 were independent of cell cycle progression.

IGF-I receptor modulation of survivin expression

To determine the signaling requirements of IGF-1 modulation of survivin, we used mouse embryonic fibroblasts (MEF) derived from IGF-1 receptor (IGF-IR) null mice, which were stably transfected with wild type (WT) or mutant IGF-IR (Romano *et al*., 1999). Stimulation with IGF-1 resulted in increased survivin expression only in cells expressing WT IGF-IR (Figure 2a). In contrast, IGF-1 stimulation of cells expressing mutant receptor GR35 or GR48 did not modulate endogenous survivin levels (Figure 2a). In addition, IGF-1 stimulation of cells expressing WT IGF-IR resulted in strong receptor tyrosine phosphorylation (Figure 2b). In contrast, tyrosine phosphorylation was detected in GR35 cells, but completely absent in GR48 cells, and no receptor phosphorylation was observed without IGF-1 (Figure 2b). In addition, cells transfected with WT IGF-IR exhibited a transformed phenotype as judged by colony formation in soft agar, whereas expression of mutant receptor GR35 or GR48 did not result in colony formation (Figure 2c).

IGF-1 induction of survivin does not involve promoter activity or changes in protein stability

To investigate the mechanism(s) of IGF-1 induction of survivin, we first analysed potential changes in survivin mRNA levels in untreated or stimulated cultures, by semiquantitative polymerase chain reaction (PCR). In control experiments, addition of FBS to serum-deprived DU145 cells resulted in increased survivin mRNA expression (Figure 3a). In contrast, IGF-1 stimulation did not significantly increase survivin mRNA levels, as compared with untreated cultures (Figure 3a). We then transfected cells with survivin promoter-luciferase constructs pLuc-441 and pLuc-1430 encompassing most of the known transcriptional requirements for *survivin* gene expression. FBS stimulation of serum-deprived DU145 cells resulted in a four to sixfold increase in β-galactosidase-normalized luciferase activity of both pLuc-441 and pLuc-1430 (Figure 3b), consistent with previous observations (Li and Altieri, 1999). In contrast, concentrations of IGF-1 that maximally increased survivin protein levels did not significantly enhance the transcriptional activity of either survivin promoter, as compared with control cultures (Figure 3b). We next asked whether IGF-1 stimulation affected survivin protein stability. Exposure of serum-deprived DU145 cells to

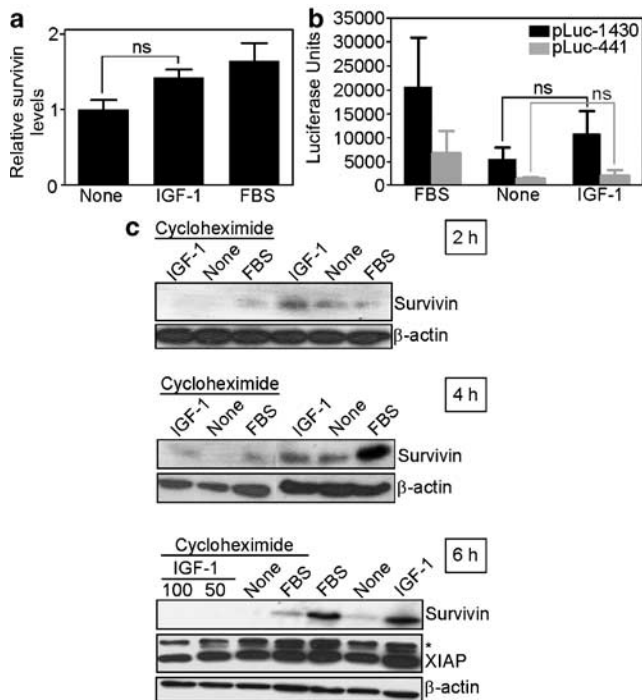

Figure 3 Requirements for IGF-1 induction of survivin. (**a**) Semiquantitative PCR. Serum-deprived DU145 cells were stimulated with medium (None), IGF-1 (100 ng/ml) or 10% FBS, and analysed for changes in survivin mRNA levels by GAPDH-normalized semiquantitative PCR. Data are the mean ± s.d. of two independent experiments. NS, not significant. (**b**) Promoter analysis. DU145 cells were co-transfected with survivin-luciferase promoter constructs pLuc-441 and pLuc-1430, stimulated with IGF-1 (100 ng/ml) or 10% FBS for 12 h and analysed for β-galactosidase-normalized luciferase activity. Data are the mean ± s.d. of two independent experiments. NS, not significant. (**c**). Effect of cycloheximide on survivin levels. Serum-deprived DU145 cells were stimulated with IGF-1 (ng/ml) or 10% FBS, incubated with cycloheximide and analysed at the indicated time intervals by immunoblotting. *nonspecific.

cycloheximide resulted in rapid disappearance of survivin levels, detected as early as 2 h after addition and throughout a 6-h time interval (Figure 3c). Although serum stimulation partially restored survivin levels in the presence of cycloheximide, treatment with IGF-1 had no effect (Figure 3c). In control experiments, IGF-1 did not affect XIAP expression in the presence or absence of cycloheximide (Figure 3c).

IGF-1 regulation of rapamycin-sensitive survivin mRNA translation

We next asked whether downstream inhibition of IGF-1 signaling by rapamycin affected survivin expression. Stimulation of serum-deprived DU145 cells with IGF-1 or FBS strongly increased survivin expression (Figure 4a), in agreement with the data presented above. Treatment of DU145 cells with rapamycin nearly completely abolished IGF-1 induction of survivin, whereas FBS stimulation of survivin was not significantly affected (Figure 4b). No modulation of XIAP was observed after IGF-1 or FBS stimulation, with or without rapamycin (Figure 4b), and no decrease in cell viability was observed at the concentrations of rapamycin used (see below, Figure 6a, left panel).

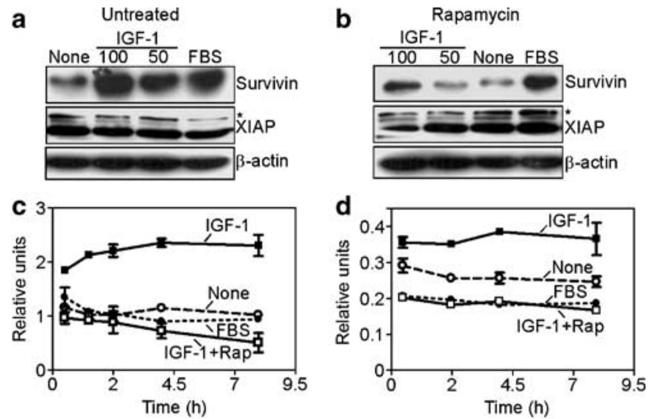

Figure 4 Modulation of IGF-1 induction of survivin by rapamycin. (**a**, **b**) Effect of rapamycin on survivin induction by IGF-1. Serum-deprived DU145 cells were stimulated with the indicated concentrations of IGF-1 (ng/ml) or 10% FBS, maintained in the absence (**a**) or presence (**b**) of rapamycin and analysed by immunoblotting after 24 h. *nonspecific. (**c**, **d**) Modulation of survivin levels after transcriptional inhibition. Serum-deprived DU145 cells were treated with the transcriptional inhibitors, actinomycin D (**c**), or DRB (**d**), stimulated with 10% FBS or IGF-1 (100 ng/ml) in the presence or absence of rapamycin and analysed at the indicated time intervals for GAPDH-normalized survivin mRNA expression by semiquantitative RT-PCR. Data are the mean ± s.d. of two independent experiments.

Next, we treated IGF-1 or FBS-stimulated DU145 cells with the transcriptional inhibitor actinomycin D, and analysed changes in survivin mRNA levels, with or without rapamycin. In these experiments, IGF-1 stimulation was associated with persistence of survivin mRNA levels over an 8-h time interval, in a reaction completely abolished by rapamycin (Figure 4c). In contrast, FBS treatment did not preserve survivin mRNA levels in the presence of actinomycin D (Figure 4c). Preservation of survivin mRNA levels in a rapamycin-sensitive response was also observed after treatment with an unrelated transcriptional inhibitor, DRB (Figure 4d), thus ruling out that the observed effect was due to nonspecific activation of a cellular stress response.

Requirement of p70S6K in IGF-1 regulation of survivin

To identify the molecular requirements of rapamycin-sensitive modulation of survivin, we transfected DU145 cells with a cDNA encoding WT or rapamycin-insensitive p70S6K, a downstream substrate of mTOR. Expression of WT p70S6K substituted for IGF-1 stimulation in inducing survivin expression in serum-deprived DU145 cells (Figure 5a). Treatment of transfected DU145 cells with rapamycin partially reduced the extent of survivin induction by p70S6K (Figure 5a). Conversely, transfection of serum-deprived DU145 cells with rapamycin-insensitive p70S6K (T389ΔCT) resulted in strong upregulation of survivin in the absence of IGF-1, in a reaction unaffected by rapamycin (Figure 5b). In control experiments, FBS induction of survivin was

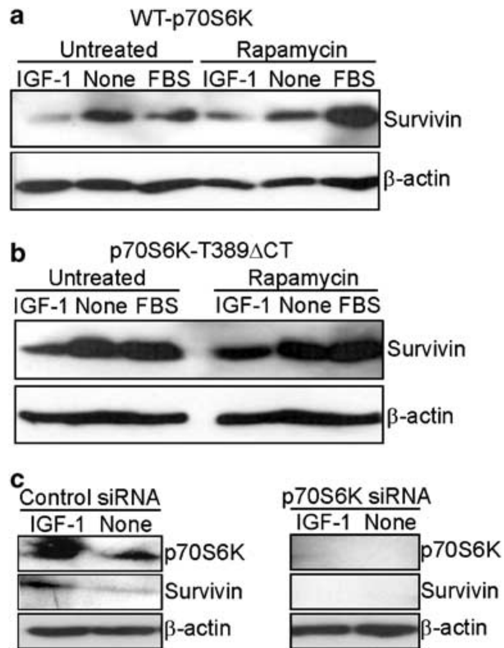

Figure 5 Requirement for p70S6K in IGF-1 modulation of survivin. Serum-deprived DU145 cells were transfected with WT (**a**) or T389ΔCT mutant (**b**) p70S6K cDNA, stimulated with IGF-1 (100 ng/ml) and analysed in the presence or absence of rapamycin by immunoblotting after 24 h. (**c**) siRNA silencing of p70S6K. Serum-deprived DU145 cells were transfected with control dsRNA oligonucleotide (left panel) or p70S6K-directed siRNA (right panel), treated with IGF-1 (100 ng/ml) or 10% FBS, and analysed by immunoblotting after 48 h.

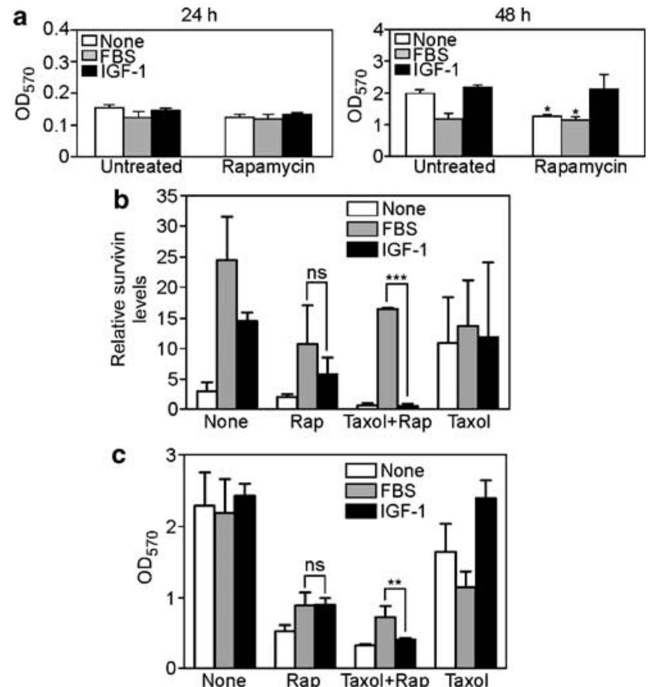

Figure 6 Effect of mTOR targeting on DU145 cell viability. (**a**) Rapamycin treatment. Serum-deprived DU145 cells were left untreated or treated with rapamycin, stimulated with 10% FBS or IGF-1 (100 ng/ml) and analysed for cell viability by MTT after 24 or 48 h. $*P = 0.021–0.032$. Data are the mean ± s.e.m. of triplicates of a representative experiment out of at least two independent determinations. (**b**) Modulation of survivin expression by taxol–rapamycin combination. Serum-deprived DU145 cells were treated with the combination of taxol plus rapamycin, harvested after 48 h and *β*-actin-normalized survivin levels were determined by quantitative immunoblotting and densitometry. NS, not significant; $***P = 0.0004$. Data are the mean ± s.d. of two independent experiments. (**c**) Cell viability. The experimental conditions are as in (**b**), except that DU145 cells were analysed for cell viability by MTT. NS, not significant; $P = 0.0046$. Data are expressed as mean ± s.e.m. of replicates of one experiment out of at least two independent determinations.

unaffected by WT or rapamycin-insensitive p70S6K in the presence or absence of rapamycin (Figure 5a and b). Next, we ablated p706SK by small interfering RNA (siRNA), and analysed changes in survivin expression with or without IGF-1 stimulation. Serum-deprived DU145 cells transfected with control siRNA exhibited increased survivin expression after IGF-1 stimulation, as compared with untreated cultures (Figure 5c). In contrast, acute knockdown of p70S6K by siRNA completely abolished IGF-1-mediated increased survivin expression (Figure 5c). Conversely, siRNA knockdown of p70S6K did not affect FBS induction of survivin in DU145 cells (not shown).

Regulation of prostate cancer cell viability by mTOR/survivin signaling

To determine the impact of IGF-1 induction of survivin in prostate cancer cells, we treated DU145 cells with rapamycin, and investigated changes in cell viability. Exposure of serum-deprived DU145 cells to rapamycin did not induce loss of cell viability for the first 24 h (Figure 6a). However, a 48-h treatment of DU145 cells with rapamycin resulted in ~50% decrease in cell viability, in a reaction fully reversed by IGF-1 stimulation, whereas FBS stimulation was ineffective (Figure 6b). Second, we combined rapamycin with established cytotoxic agents and investigated changes in survivin expression and cell viability. Treatment of DU145 cells with the combination of rapamycin plus a suboptimal concentration of taxol for 24 h resulted in nearly complete disappearance of survivin expression, as compared with either treatment alone (Figure 6b). In contrast, the combination of doxorubicin plus rapamycin was ineffective (not shown). IGF stimulation of DU145 cells treated with the combination of taxol plus rapamycin failed to restore survivin expression, whereas FBS increased survivin levels to approximately 50% of those of untreated cultures (Figure 6b). Consistent with a critical reduction in the antiapoptotic threshold maintained by survivin, the taxol plus rapamycin combination was more effective than either treatment alone in reducing DU145 cell viability in the presence of IGF-1 (Figure 6c).

Discussion

In this study, we have shown that IGF-1 stimulation of prostate cancer cells results in increased survivin expression, and that this pathway is mediated by stabilization and translation of a pool of survivin mRNA. Inhibition

of mTOR (Wullschleger *et al*., 2006) with rapamycin, alone or in combination with taxol, or molecular or genetic interference with its downstream target, p70S6K (Wullschleger *et al*., 2006), abolished survivin increase by IGF-1, and decreased prostate cancer cell viability.

Because of its 'crossroad' role in multiple essential pathways of tumor cell maintenance, and its differential expression in cancer as opposed to normal tissues, survivin is being actively pursued as a novel target for rational cancer therapy (Altieri, 2003). A validating principle of this approach is that lowering intracellular survivin levels below a critical threshold using a variety of approaches, including antisense, dominant negative mutants or siRNA sequences, has been consistently associated with arrest of cell proliferation, spontaneous apoptosis and sensitization to cell death stimuli, including cytotoxics and ionizing radiation (Altieri, 2006). In this context, much attention has been devoted towards elucidating the molecular requirements of *survivin* gene transcription, but recent evidence points to additional, non-transcriptional mechanisms controlling survivin levels in tumor cells (Altieri, 2003). One such pathway is centered on IGF-1 ligation to its cognate membrane receptor, which has been shown to increase survivin expression in prostate (Zhang *et al*., 2005), myeloma (Stromberg *et al*., 2006) and liver (Hopfner *et al*., 2006) tumor cell types, even though the underlying mechanism(s) of this response had remained elusive. As now reported here, this pathway depends on IGF-IR transforming potential (Sell *et al*., 1994), does not involve changes in cell cycle distribution and is not associated with *de novo* survivin promoter activity or increased survivin protein stability. Conversely, IGF-1 modulates survivin levels by favoring stabilization and translation of a survivin mRNA pool through activation of the mTOR pathway.

Regulation of survivin expression has been linked in multiple experimental systems to increased Akt activity, a Ser/Thr kinase downstream of PI3 kinase signaling (Amaravadi and Thompson, 2005). This is relevant for prostate cancer, where survivin is highly expressed (Fornaro *et al*., 2003; Krajewska *et al*., 2003), and Akt activity is commonly deregulated after loss of the inhibitory lipid phosphatase, phosphatase and tensin homolog deleted on chromosome 10 (Majumder and Sellers, 2005). In turn, increased Akt activity provides a broad antiapoptotic environment through cytoplasmic trapping of cell death mediators (Datta *et al*., 1997; Goswami *et al*., 2005), modulation of NFκB (Kane *et al*., 2002, 1999) and repression of apoptotic inducers (Brunet *et al*., 1999). In addition, Akt phosphorylates and inactivates the tuberous sclerosis complex, TSC2, a negative regulator of mTOR signaling (Wullschleger *et al*., 2006). The data presented here identify a novel survival circuitry initiated by IGF-1/Akt signaling, and involving mTOR activation, p70S6K activation and increased stability/translation of a survivin mRNA pool. This pathway is specific for IGF-1, as serum mitogens upregulated survivin in tumor cells independently of mTOR or p70S6K, and is centered on survivin, as XIAP (Salvesen and Duckett, 2002), a IAP member whose levels are controlled by protein translation (Holcik *et al*., 2000), was not affected.

The mTOR pathway constitutes a 'sensor' network for environmental nutrients or stress conditions, thus affecting cell growth (cell volume), cellular proliferation and activation of metabolic rescue pathways, that is, autophagy (Wullschleger *et al*., 2006). As a multifaceted mTOR effector, survivin appears ideally positioned to contribute to these responses, by favoring mitotic progression, resistance to apoptosis and increased cellular adaptation to stress (Altieri, 2003). Conversely, targeted inhibition of IGF-1/Akt/mTOR couples to cell cycle arrest and induction of apoptosis, which may be contributed by acute loss of survivin levels (Decker *et al*., 2003; Hopfner *et al*., 2006; Stromberg *et al*., 2006), Forkhead-mediated transcription of the cyclin-dependent kinase inhibitor p27 (Wullschleger *et al*., 2006) and activation of p53-dependent apoptosis (Levine *et al*., 2006).

In summary, these data reinforce the rationale of pharmacologic inhibition of IGF-1/Akt/mTOR signaling for prostate cancer therapy in humans (Hay, 2005), and suggests that ablation of survivin mRNA translation may provide an additional strategy to remove an antiapoptotic mechanism potentially contributing to aggressive tumor behavior, *in vivo*.

Materials and methods

Cell culture conditions

Prostate adenocarcinoma DU145 and cervical carcinoma HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). DU145 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 1 mM sodium pyruvate, 10% heat inactivated FBS plus 100 U Pen–Strep antibiotic mixture and maintained in a 5% CO_2 incubator at 37°C, as described (Fornaro *et al*., 2003). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). IGF-1 was purchased from R&D System (Minneapolis, MN, USA). Rapamycin, cycloheximide and the transcriptional inhibitors, actinomycin D and 5,6 dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were from Sigma (St Louis, MO, USA). All working solutions were prepared in phosphate-buffered saline, pH 7.4. For stimulation, 4×10^6 DU145 cells were seeded in six-well plates in 2 ml of serum-free DMEM for 24 h, and incubated with increasing concentrations of IGF-1 for 12–48 h in the presence or absence of rapamycin (20 nM). When incubation reactions were prolonged to 48 h, IGF-1 and rapamycin were replaced daily in fresh medium. MEF from IGF-IR null mice (R−), or MEF transfected with human WT IGF-IR were cultured as described (Sell *et al*., 1994). R− cells were transduced with retroviral particles expressing IGF-IR mutants GR35 and GR48, and stable cell lines were selected using geneticin (1 mg/ml). Briefly, GR35 contains a mutant IGF-IR in which four serine residues at position 1280–1283 were changed to alanine (Li *et al*., 1996). GR48 contains an IGF-IR with a triple tyrosine mutation to phenylalanine of residues 1131, 1135 and 1136 (Romano *et al*., 1999).

Modulation of protein and mRNA expression

After IGF-1 stimulation, DU145 cells were harvested at increasing time intervals and solubilized in 150 μl of lysis buffer containing 20 mM Tris, pH 7.2, 0.5% sodium

deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid plus 5% protease inhibitor cocktail tab (Roche, Indianapolis, IN, USA). Protein-normalized (50 μg) extracts were separated on 12% SDS polyacrylamide gels, transferred to PVDF membranes (Millipore, Billerica, MA, USA) and analysed by immunoblotting, as described (Dohi *et al*., 2004). Antibodies to β-actin (1:5000, clone AC-15, Sigma), survivin (1:1000, NOVUS Biologicals, Littleton, CO, USA), XIAP (1:500, BD Bioscience, San Jose, CA, USA) or total p70S6K (1:500, Cell Signaling, Danvers, MA, USA) were used with detection by chemiluminescence (Plus Reagents, GE Healthcare, Piscataway, NJ, USA). In some experiments, WT, GR35 and GR48 cells were plated onto 60 mm tissue culture plates (2×10^5 cells/plate) and incubated for 24 h at 37°C. After washes, cells were stimulated with or without 100 ng/ml IGF-1 for 48 h at 37°C, lysed in 20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 10 mM NaF, 1 mM $NaVO_4$, 1 mM $Na_4O_7P_2$, plus protease inhibitors, and aliquots of protein extracts (50 μg) were analysed by immunoblotting. In other experiments, R− cells transfected with WT, GR35 or GR48 mutants were lysed, and proteins were immunoprecipitated with an antibody to IGF-IR plus protein A-Sepharose, followed by immunoblotting with antibodies to IGF-IR or tyrosine phosphorylated proteins (PY20).

For analysis of mRNA stability, serum-deprived DU145 cells were stimulated with medium, IGF-1 (100 ng/ml) or 10% FBS for 6 h at 37°C. Samples were incubated with 10 μM actinomycin D, or, alternatively, 50 μM DRB, harvested at 2–8 h time intervals, and processed for total RNA extraction (RNeasy Mini, Qiagen, Valencia, CA, USA). For analysis of protein stability, DU145 cells were incubated in serum-deprived conditions for 24 h, stimulated with medium, IGF-1 or 10% FBS for 18 h, followed by incubation with 10 μM cycloheximide. Samples were harvested at 2–6 h time intervals, and protein-normalized extracts were analysed by immunoblotting.

Reverse transcription and semiquantitative PCR
Total RNA extracted from DU145 cells was reverse transcribed (1 μg) in the presence of SuperScript II polymerase plus random primers (Invitrogen). Amplification of survivin (27 cycles) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (32 cycles) cDNA was carried out by semiquantitative PCR. Products were separated on 1.5% agarose gels and band intensity was quantified using Image J software followed by GAPDH normalization.

p70S6K transfection
DU145 cells were transfected with WT or rapamycin-resistant p70S6K mutant cDNA (4 μg) using LipofectAmine 2000 (Invitrogen, 6 μl) in Opti-Mem medium (1 ml) (Invitrogen, Carlsbad, CA, USA). After 5 h, cells were incubated in serum-deprived conditions for 16 h at 37°C, stimulated with IGF-1 or 10% FBS for 24 h and analysed for changes in protein expression with or without rapamycin (20 nM), by immunoblotting. For gene silencing experiments by siRNA, DU145 cells were transfected with p70S6K-directed siRNA SMARTpool (M-003616-02-0010, Dharmacon Inc., Lafayette, CO, USA), or control non-targeted dsRNA oligonucleotide (VIII) using LipofectAmine 2000. After 5 h, the medium was replaced with serum-free DMEM with or without IGF-1 (100 ng/ml) or 10% FBS. Samples were harvested after 24 or 48 h, and analysed by immunoblotting.

Survivin promoter activity
DU145 or HeLa cells were transfected with survivin promoter constructs upstream of a luciferase reporter gene (pLuc-441 and pLuc-1430) encompassing most of the transcriptional requirements for *survivin* gene expression (Li and Altieri, 1999) plus 1 μg *LacZ* cDNA. After transfection, cells were incubated in serum-deprived conditions for 16 h, stimulated with IGF-1 or 10% FBS for 12 h and analysed for β-galactosidase-normalized luciferase activity using a DTX 880 Multimode Detector luminometer (Beckman Coulter, Fullerton, CA, USA) at A_{405}.

Analysis of cell viability and cell cycle
Serum-deprived DU145 cells were seeded in 96-wells plates for 24 h, and stimulated with medium, IGF-1 (100 ng/ml) or 10% FBS in the presence or absence of rapamycin (20 nM) for 24 or 48 h. In some experiments, serum-deprived DU145 cells were treated with the combination of rapamycin plus taxol (25 μM) for 24 h, stimulated with IGF-1 or FBS and harvested after 48 h. After addition of 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (10% of total volume, Sigma) for 4 h at 37°C, samples under the various conditions tested were centrifuged at 3000 r.p.m. for 10 min, suspended in 200 μl dimethyl sulfoxide (Sigma) for 10 min at 22°C and absorbance was determined at A_{570}. Alternatively, samples were analysed for changes in survivin levels by β-actin-normalized quantitative immunoblotting. In other experiments, serum-deprived DU145 cells stimulated with FBS or IGF-1 were stained with propidium iodide and analysed for DNA content at 12–48 h time intervals by flow cytometry (Li *et al*., 1999).

Anchorage-independent cell growth
Cell growth in soft agar was assayed by scoring the number of colonies formed in DMEM containing 0.3% agarose with a 0.5% agarose medium underlay. R−, WT, GR35 or GR48 (2×10^3) cells were seeded on 60-mm diameter plates in triplicate. Cells were fed with 1.5 ml of DMEM medium every 3 days. Colonies larger than 100 μm were counted after 14 days.

Statistical analysis
Data were analysed with two-sided unpaired t tests in the GraphPad software package for Windows (Prism version 4.0). A P-value of 0.05 was considered as statistically significant. All statistical tests were two-sided.

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