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The ubiquitin-proteasome pathway regulates survivin degradation in a cell cycle-dependent manner

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SUMMARY

Survivin, a human inhibitor of apoptosis protein (IAP), plays an important role in both cell cycle regulation and inhibition of apoptosis. Survivin is expressed in cells during the G_2/M phase of the cell cycle, followed by rapid decline of both mRNA and protein levels at the G_1 phase. It has been suggested that cell cycle-dependent expression of survivin is regulated at the transcriptional level.

In this study we demonstrate involvement of the ubiquitin-proteasome pathway in post-translational regulation of survivin. Survivin is a short-lived protein with a half-life of about 30 minutes and proteasome inhibitors greatly stabilise survivin in vivo. Expression of the *survivin* gene under the control of the CMV promoter cannot block cell cycle-dependent degradation of the protein. Proteasome inhibitors can block survivin degradation during the G_1 phase and polyubiquitinated derivatives can be detected in vivo. Mutation of critical amino acid residues within the baculovirus IAP repeat (BIR) domain or truncation of the N terminus or the C terminus sensitises survivin to proteasome degradation. Together, these results indicate that the ubiquitin-proteasome pathway regulates survivin degradation in a cell cycle-dependent manner and structural changes greatly destabilise the survivin protein.

Key words: Survivin, Cell cycle, Ubiquitin, IAP, Proteasome

INTRODUCTION

The inhibitor of apoptosis protein (IAP) family comprises a class of cell death inhibitors containing one to three BIR (baculovirus IAP repeat) domains (Deveraux and Reed, 1999). They were initially identified as baculoviral proteins that prevent the host cell from undergoing apoptosis following infection. Since then IAPs have been found in yeast, invertebrates and vertebrates. Survivin, a human IAP protein, is the smallest member of the IAP family, containing only one BIR domain. Survivin is expressed ubiquitously during embryonic and fetal development but is undetectable in most normal adult tissues (Ambrosini et al., 1997). Several studies have reported that survivin is expressed at high levels in human cancers (Ambrosini et al., 1997; Kawasaki et al., 1998; LaCasse et al., 1998; Lu et al., 1998).

Like other mammalian IAPs (e.g. XIAP, c-IAP-1, c-IAP-2) (Deveraux et al., 1998; Deveraux et al., 1997; Roy et al., 1997; Takahashi et al., 1998) survivin can bind to caspase-3 and caspase-7 (Tamm et al., 1998) and overexpression of survivin blocks apoptosis induced by caspase-3 and caspase-7, growth factor deprivation and the microtubule-stabilising agent Taxol (Ambrosini et al., 1997; Li et al., 1998; Tamm et al., 1998). There is increasing evidence to indicate that survivin is involved in the regulation of cell division (Fraser et al., 1999; Li et al., 1999; Li et al., 1998; Miller, 1999; Reed and Reed, 1999; Uren et al., 1999; Uren et al., 1998). BIR-1, a survivin homologue encoded by the *bir-1* gene in *C. elegans*, is highly expressed during embryogenesis. Inhibition of *bir-1* expression resulted in the formation of multinucleated cells rather than cell death (Fraser et al., 1999) and this defect in cytokinesis could be suppressed partially by overexpression of survivin. Similar results have been observed with mutants of *BIR1* and *bir1*, survivin homologues in yeast (Uren et al., 1999). In contrast to other mammalian IAPs, survivin is unique in localising to spindle microtubules in the G_2/M phase (Li et al., 1999; Li et al., 1998). Inhibition of survivin expression or disruption of survivin interaction with microtubules induces defective cytokinesis with hyperploidy, multipolar mitotic spindles, supernumerary centrosomes and consequent apoptosis at G_2/M phase (Li et al., 1999). This indicates that survivin is a regulator of cell division and is required to protect cells from apoptosis during mitosis.

The ubiquitin-proteasome pathway plays a central role in the regulation of essential cellular processes such as cell cycle control, antigen processing, transcription and signal transduction. Many short-lived, key regulator proteins including cyclins (cyclin A, B, D, E) and cyclin kinase inhibitors CKI (p21, p27, p57) (King et al., 1996; Koepp et al., 1999; Shirane et al., 1999), transcription factors E2F-1

(Hateboer et al., 1996), IκB (Chen et al., 1996), p53 (Scheffner et al., 1990), SMAD2 (Lo and Massagu, 1999; Zhu et al., 1999), c-Jun (Treier et al., 1994) and β-catenin (Aberle et al., 1997; Orford et al., 1997) are regulated by ubiquitination. Two steps are involved in ubiquitin-proteasome-dependent degradation of proteins: labelling of the target protein with multiple ubiquitin moieties and degradation of the tagged protein by the 26S proteasome (Ciechanover, 1998; Laney and Hochstrasser, 1999). The 76-amino-acid ubiquitin polypeptide (Ub) is joined to target proteins via reversible isopeptide linkages between the carboxy terminus of ubiquitin and lysine side chains in the target proteins. Free Ub is recruited by the E1 Ub-activating enzyme and subsequently transferred to the E2 Ub-conjugating enzyme. With the help of the E3 Ub-protein ligases, which are responsible for substrate recognition, ubiquitin is then attached to the target protein. Polyubiquitinated proteins are recognised by the 26S proteasome complex and rapidly degraded into short peptides.

Expression of survivin is cell cycle-dependent with upregulation in G_2/M and rapid decline in G_1 (Li et al., 1998). As for several other proteins involved in the control of the cell cycle (King et al., 1996), the temporal pattern of survivin expression suggests that the ubiquitin-proteasome pathway might also be involved in the regulation of survivin.

In this study, we demonstrate that the ubiquitin-proteasome pathway regulates survivin degradation in a cell cycle-dependent manner and structural changes greatly destabilise the survivin protein.

MATERIALS AND METHODS

Chemicals and reagents

Proteasome inhibitors MG132 (N-CBZ-leu-leu-leucinal) and Calpain Inhibitor I, ALLN (N-acetyl-leu-leu-norleucinal) were purchased from Sigma and used at final concentrations of 20 μM and 100 μM, respectively. PMSF and leupeptin were purchased from Sigma and used at final concentrations of 200 μM and 200 μg/ml, respectively. Lactacystin was purchased from Calbiochem and used at a final concentration of 12.5 μM. Cycloheximide was purchased from Sigma and used at a final concentration of 50 μg/ml. Taxol was purchased from Sigma and used at a final concentration of 1 μM. Affinity-purified rabbit anti-human survivin antibody, which recognises amino acids 3-19 of human survivin, was purchased from R&D Systems Inc. Anti-ubiquitin antibody (sc-8017) was purchased from Santa Cruz Biotechnology Inc. Anti-V5 antibody was purchased from Invitrogen.

Cell-cycle synchronisation

Human embryonal kidney 293 cells were treated with 400 μM L-mimosine (Sigma), 2 mM thymidine (Sigma) or 40 ng/ml nocodazole (Calbiochem) for 12-16 hours. Cell cycle status was analysed with a FACSCalibur flow cytometer (Becton Dickinson) by measuring fluorescence from cells stained with propidium iodide.

Plasmids and transfection

Full-length human survivin cDNA was generated by RT-PCR and subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and the pcDNA3.1/V5/His-TOPO vector (Invitrogen) to generate pc-survivin and survivin/V5/His (containing V5 and His tags at the C terminus), respectively. Survivin mutants Cys 84→Ala (C84A) and His 77→Ala (H77A) were generated by PCR-based mutagenesis and subcloned into pcDNA3. A survivin mutant truncated at the C terminus to encode residues M1-V89 was generated by PCR and subcloned into pcDNA3, and designated as pc-survivin-ΔC. A survivin mutant truncated at the N terminus to encode residues D71-D142 was generated by PCR and subcloned into pcDNA3.1/V5/His-TOPO, and designated as survivin-ΔN/V5/His. The HA-tagged ubiquitin plasmid, pMT123, was a gift from Dr Dirk Bohmann (Treier et al., 1994). His-myc-tagged ubiquitin plasmids pCW7 (wild type) and pCW8-K48R (mutant) were kindly provided by Dr Ron R. Kopito (Ward et al., 1995). 293 cells were transiently transfected with plasmids using a Calcium Phosphate Transfection kit (Eppendorf).

TAP-tagged survivin was generated by cloning the TAP-tag (Rigaut et al., 1999) at the C terminus of survivin. The resulting construct was cloned into pNRTIS-33 expression vector (Tenev et al., 2000) and HeLa stable cell lines, selected with G-418 were generated by transfection with Superfect™ (Qiagen).

Immunoblotting assay

Cells were lysed in 1% Triton buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100) plus the protease inhibitor cocktail Complete™ (Boehringer Mannheim). After centrifugation at 13,000 ***g*** for 15 minutes at 4°C, supernatant was recovered and protein concentrations were determined using BCA Protein Assay Reagents (Pierce). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and resolved proteins were transferred onto Immobilon-P membranes (Millipore) for probing with the various primary antibodies. Antigen-antibody complex was visualized using the ECL™ system (Amersham).

Cycloheximide inhibition

Half-life experiments employing cycloheximide inhibition of protein synthesis were performed as described previously (Patrick et al., 1998). Proteasome inhibitors were added 1 hour before cycloheximide treatment and cell lysates were subjected to SDS-PAGE and immunoblotting with anti-survivin antibody.

Preparation of cell extracts

After being synchronised as described previously, cells were washed with phosphate-buffered saline, resuspended in distilled water, and freeze-thawed three times. Cell extracts were subjected to centrifugation at 100,000 ***g*** for 4 hours at 4°C, and the supernatant (S100Pr-) was recovered and used for in vitro ubiquitination assays.

In vivo ubiquitination assay

293 cells were transiently transfected with pc-survivin or cotransfected with pc-survivin and one of the ubiquitin expression vectors. 24-36 hours after transfection, cells were cultured with or without proteasome inhibitors for 12-16 hours. Cells were lysed in 1× SDS sample buffer and cell lysates were boiled for 5 minutes. DNA was sheared by repeated passage of the sample through a 21-G needle. After centrifugation at 13,000 ***g*** for 10 minutes, supernatants were subjected to SDS-PAGE and immunoblotting with anti-survivin antibody.

Nickel affinity chromatography

293 cells were transfected with survivin/V5/His alone or cotransfected with HA-tagged ubiquitin plasmid pMT123. 36 hours after transfection, cells were treated with MG132 overnight. His-tagged survivin was purified through Ni-NTA (Affiniti) agarose as described previously (Janknecht et al., 1991). Briefly, cells were resuspended in buffer A (10 mM Hepes, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 mM NaCl, 1 mM dithiothreitol, 10 mM NaF, plus the cocktail of protease inhibitors). After three cycles of freeze-thaw, cell debris was pelleted (10,000 ***g*** for 10 minutes) and the supernatant was incubated with Ni-NTA agarose for 1 hour. After three washes, His-tagged protein was eluted with buffer containing 200 mM imidazole and subjected to SDS-PAGE and immunoblotting with anti-ubiquitin antibody.

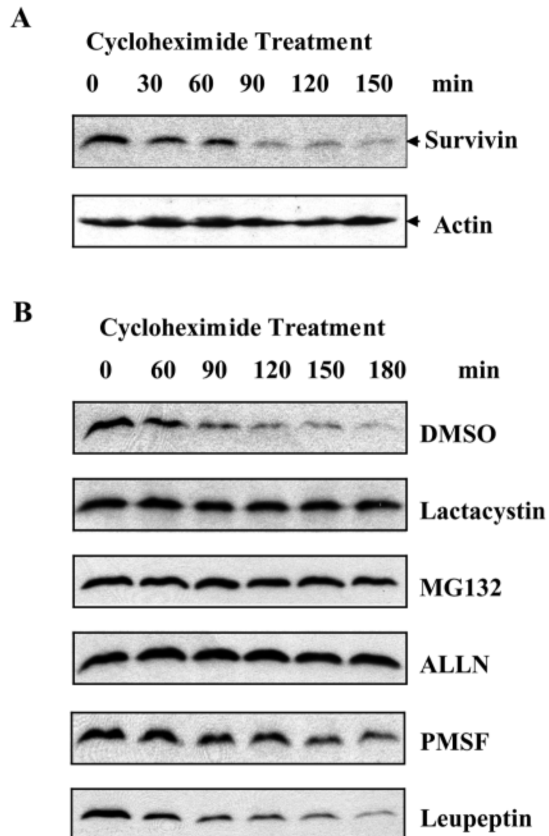

Fig. 1. Survivin is a short-lived protein and proteasome inhibitors stabilise endogenous survivin. (A) 293 cells were treated with 50 μg/ml cycloheximide. After the indicated times cells were lysed in 1% Triton buffer. Equal amounts of protein were subjected to immunoblotting with anti-human survivin antibody and anti-actin antibody. (B) 293 cells were pretreated with 5 μl/ml DMSO, 12.5 μM lactacystin, 20 μM MG132, 100 μM ALLN, 200 μM PMSF or 200 μg/ml leupeptin for 1 hour and cycloheximide treatment was performed as described earlier. Cell lysates were subjected to immunoblotting with anti-human survivin antibody.

RESULTS

Survivin is a short-lived protein and proteasome inhibitors stabilise endogenous survivin

We determined the half-life ($t_{1/2}$) of survivin in 293 cells by cycloheximide treatment. Cycloheximide interacts with the translocase enzyme and blocks protein synthesis in eukaryotic cells. After cycloheximide treatment, the level of survivin protein quickly dropped with about half of the protein degraded after 30 minutes (Fig. 1A, upper panel). No reduction was found in the level of actin used as a control (Fig. 1A, lower panel). This result shows that survivin is rapidly turned over with a $t_{1/2}$ of about 30 minutes.

To determine the proteases involved in the degradation of survivin, we treated cells with three different kinds of proteasome inhibitors (lactacystin, MG132, ALLN) and the protease inhibitors PMSF and leupeptin 1 hour before blocking protein synthesis with cycloheximide. Lactacystin is a specific inhibitor of the 20S proteasome (Fenteany et al., 1995), while MG132 and ALLN are potent proteasome inhibitors but less specific. PMSF is a serine protease inhibitor and leupeptin is an inhibitor of trypsin-like and cysteine proteases. Degradation of survivin was blocked by pretreatment with proteasome inhibitors but not protease inhibitors (Fig. 1B). These results indicate that survivin is a short-lived protein and proteasomes are involved in its degradation.

Proteasomes regulate survivin degradation at G_1 phase

L-mimosine was used to block the cell cycle in G_1, thymidine was used to block the cell cycle in S phase and nocodazole was used to block the cell cycle at G_2/M (confirmed by flow cytometry, Fig. 2A). As has been reported previously (Li et al., 1998) the level of endogenous survivin was increased after nocodazole treatment and greatly reduced after L-mimosine treatment (Fig. 2B). We next examined whether the sharp decline of survivin protein level in the G_1 phase was due to degradation by proteasomes. We treated cells with three different proteasome inhibitors MG132, ALLN and lactacystin. The reduction in levels of survivin protein in the G_1 phase was abolished in the presence of any of the three proteasome inhibitors (Fig. 2C). These results indicate that the decline of survivin protein level in G_1 phase is a result of proteasome activity.

Expression of survivin under control of the CMV promoter does not block survivin degradation at G_1 phase

Several reports have shown that survivin expression is regulated at the transcriptional level with upregulation at G_2/M and downregulation at G_1 phase (Kobayashi et al., 1999; Li et al., 1998). However, post-transcriptional cell cycle-dependent regulation of survivin degradation has not been previously reported. To check for the presence of such regulation at the protein level we generated a stable HeLa cell line expressing survivinTAP-tag under control of the tetracycline-responsive transcriptional activator (tTA) regulated CMV promoter (Tenev et al., 2000). We selected a clone expressing survivinTAP-tag at a level close to that of endogenous survivin. Cells were arrested with L-mimosine, thimidine and nocodazole as described above and the distribution of endogenous survivin and survivinTAP-tag at different stages of the cell cycle were analysed by western blot. As is shown in Fig. 3, constitutive CMV promoter-driven expression of survivinTAP-tag cDNA did not block the decline in the level of survivin at G_1. The levels of survivinTAP-tag paralleled those of endogenous survivin, maximal at G_2/M, followed by rapid degradation at G_1. This result clearly shows the presence of a post-transcriptional cell cycle-dependent mechanism of survivin regulation in vivo.

In vivo ubiquitination

To determine the involvement of ubiquitination in survivin degradation in vivo, we transfected 293 cells with an expression vector encoding wild-type survivin. After overnight incubation with or without the proteasome inhibitors MG132, ALLN and lactacystin, cells were lysed under strongly denaturing conditions to inhibit isopeptidases (Lo and Massagu, 1999). Cell lysates were subjected to immunoblotting with anti-survivin antibody. High-molecular-mass survivin products accumulated in cells treated with

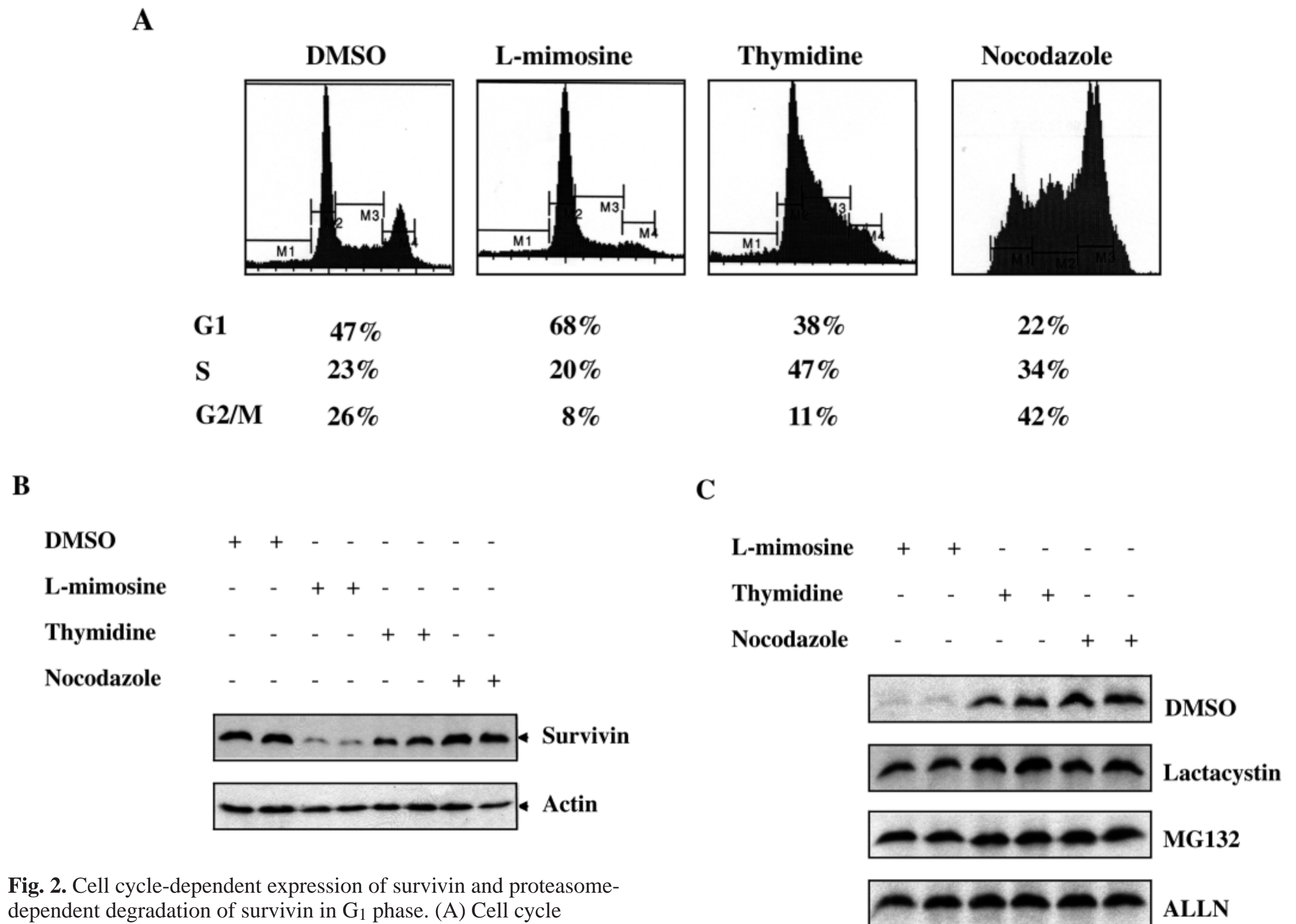

Fig. 2. Cell cycle-dependent expression of survivin and proteasome-dependent degradation of survivin in G_1 phase. (A) Cell cycle synchronisation was achieved by treatment of 293 cells with 5 μl/ml DMSO, 400 μM L-mimosine, 2 mM thymidine or 40 ng/ml nocodazole for 12-16 hours. Cell cycle distribution was analysed using a FACSCalibur flow cytometer. (B) 293 cells treated under the same conditions as in A were lysed in 1% Triton buffer. Equal portions of protein were subjected to immunoblotting with anti-human survivin antibody or anti-actin antibody. (C) 293 cells were pretreated with 5 μl/ml DMSO, 12.5 μM lactacystin, 20 μM MG132 or 100 μM ALLN for 2 hours and cell cycle synchronisation was performed as described in A. Equal amounts of protein were subjected to immunoblotting with anti-human survivin antibody.

proteasome inhibitors but not in cells left untreated (Fig. 4A). Coexpression of survivin with ubiquitin led to a moderate increase in ubiquitinated survivin (Fig. 4B, lane 4) and again the accumulation of ubiquitinated survivin was enhanced by proteasome inhibitors (Fig. 4B, lane 5-7).

More direct evidence for in vivo ubiquitination of survivin came from nickel affinity chromatography. 293 cells were transfected with His-tagged survivin or cotransfected with the tagged survivin plus HA-tagged ubiquitin. After treatment with the proteasome inhibitor MG132 overnight, cell lysates were subjected to Ni-NTA agarose purification, and His-tagged proteins were eluted and subjected to anti-Ub immunoblotting to reveal ubiquitin-conjugated survivin products. Polyubiquitinated survivin products were detected only in cells cotransfected with His-tagged survivin and Ub but not in untransfected cells and cells transfected with His-tagged survivin or Ub alone (Fig. 4C).

Taken together, these data strongly suggest that survivin is ubiquitinated in vivo and degraded by the ubiquitin-proteasome pathway.

Survivin is ubiquitinated at several lysine residues

To determine which lysine residues of survivin mediate its ubiquitination we generated several point mutants in which a lysine residue is replaced by arginine. However, cotransfection of mutants with Ub-expressing plasmids revealed that single mutations are not able to block ubiquitination (not shown). These results show that survivin is most probably ubiquitinated at several lysine residues. To investigate this hypothesis we analysed the effect of the K48R ubiquitin mutant on survivin ubiquitination. Normally a polyubiquitin chain is produced by linkage of additional ubiquitin moieties to Lys 48 of a previously attached ubiquitin molecule. The K48R mutant has a dominant negative effect on polyubiquitination and degradation by terminating the elongation of polyubiquitin chains (Ward et al., 1995). Cotransfection of the K48R mutant produced higher levels of products ubiquitinated at multiple sites in both pc-survivin and survivin/V5/His transfected cells (Fig. 5A, lanes 5,11 and B, lanes 3,4). The production of several distinct ubiquitinated forms of survivin under these conditions indicates that these conjugates most likely represent

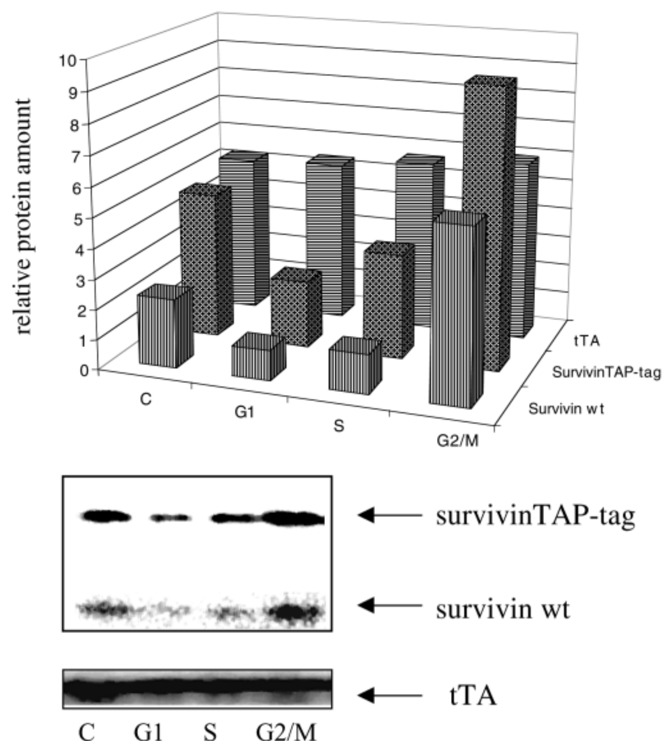

Fig. 3. Post-transcriptional cell cycle-dependent regulation of survivin. A stable HELA cell line expressing survivinTAP-tag cDNA under the control of tTA regulated CMV promoter was generated. Cell cycle synchronisation was achieved by treatment of the cells with 5 μl/ml DMSO (C), 400 μM L-mimosine (G_1), 2 mM thymidine (S) or 40 ng/ml nocodazole (G_2/M) for 12-16 hours. Cells were lysed in 1% Triton buffer and the lysates were subjected to immunoblotting with anti-human survivin antibody. The relative density of ECL signals was determined on a Molecular Imager (Bio-Rad Laboratories, CA94547).

attachment of mono-K48RUb moieties at multiple lysine residues. Two truncated survivin mutants, survivin-ΔC(M1-V89) and survivin-ΔN/V5/His(D71-D142), were analysed by cotransfection with K48R mutant. Both mutants showed reduced levels of ubiquitination (Fig. 5A, lane 12 and B, lane 8), but with more extensive reduction when the N terminus was truncated. These results indicate that ubiquitination most probably occurs at several lysine residues.

Deletion mutants or point mutations within the BIR domain greatly sensitise survivin to ubiquitin-proteasome degradation

Transfection experiments have shown that the survivin-ΔN/V5/His mutant (in which the BIR domain is disrupted) and the survivin-ΔC mutant (in which the C-terminal tail, critical for microtubule binding, is lost) both have reduced stability (Fig. 5A, lanes 2,4,6 and B, lanes 5,7). Addition of the proteasome inhibitor MG-132 was able to stabilise both mutants (Fig. 5A, lanes 8,10,12 and B, lanes 6,8). These results indicate that both the N-terminally located BIR domain and the coiled-coil tail are crucial for stability of the protein.

IAPs are zinc-binding proteins. The nuclear magnetic resonance (NMR) structure of the BIR2 domain of XIAP resembles a classical zinc finger in which amino acids C200, C203, H220 and C227 are important for chelating zinc and stabilising protein folding (Sun et al., 1999). Amino acids H77 and C84 of survivin (which correspond to H220 and C227 of XIAP) are within the BIR domain and mutation of these amino acids would be predicted to disrupt the anti-apoptotic function of survivin (Li et al., 1998). To investigate further the role of the BIR domain in mediating protein stability, we generated two further survivin mutants, H77A and C84A. The levels of C84A and H77A in transfectants were very low compared to wild-type survivin (Fig. 6, lanes 2,3,9). After treatment with proteasome inhibitors, their levels increased and reached the level of wild-type survivin (Fig. 6, lanes 4-7,10). These results indicate that survivin BIR domain mutants, with disrupted anti-apoptotic function (Li et al., 1998), are unstable in the cell and degrade rapidly. In contrast, Taxol, which stabilises microtubules and blocks the cell cycle at G_2/M phase, had no effect on the level of expression of these mutants (Fig. 6, lanes 8,11). This indicates that the degradation of C84A and H77A is through the proteasome pathway, and blocking cells in G_2/M or stabilisation of microtubules does not protect them from degradation. We also observed an enhanced level of polyubiquitinated C84A and H77A products after MG132 treatment, which confirms faster degradation of these mutants (data not shown). The accelerated degradation of these various survivin mutants shows that the BIR domain and the coiled-coil tail are crucial for maintaining the stability of survivin during G_2/M phase.

DISCUSSION

The ubiquitin-proteasome pathway is important in spatial and temporal regulation of the cell cycle through elimination of proteins regulating cell cycle progression and checkpoints, and seems to be particularly important in progression through mitosis (King et al., 1996; Pines, 1999). Like its homologues in *C. elegans* and yeast, the human IAP protein survivin serves as a cytokinesis regulator (Li et al., 1999; Li et al., 1998). Disruption of survivin function causes a severe disorder in cytokinesis, followed by formation of polyploid cells and development of apoptosis (Li et al., 1999). Expression of survivin is cell cycle-dependent with rapid turnover in the G_1 phase. It has been shown that the G_1 transcriptional repressor elements within the survivin promoter contribute to cell cycle-dependent expression of the gene (Li et al., 1998). Here we demonstrate firstly that the ubiquitin-proteasome pathway is involved in the degradation of survivin in a cell cycle-dependent manner, and secondly that both the BIR domain and the C-tail of survivin are crucial for maintaining protein stability at G_2/M.

Firstly, proteasome inhibitors prolonged the half-life of survivin in vivo. Lactacystin is a specific 20S proteasome inhibitor with no effect on cysteine proteases, serine proteases, trypsin and chymotrypsin even upon extended exposure (Fenteany et al., 1995). ALLN and MG132 are also potent proteasome inhibitors but less specific, with additional inhibitory activities against cysteine proteases and calpain. After blocking protein synthesis with cycloheximide, the level of survivin become stable upon treatment with proteasome inhibitors. Stabilisation of survivin in vivo by proteasome

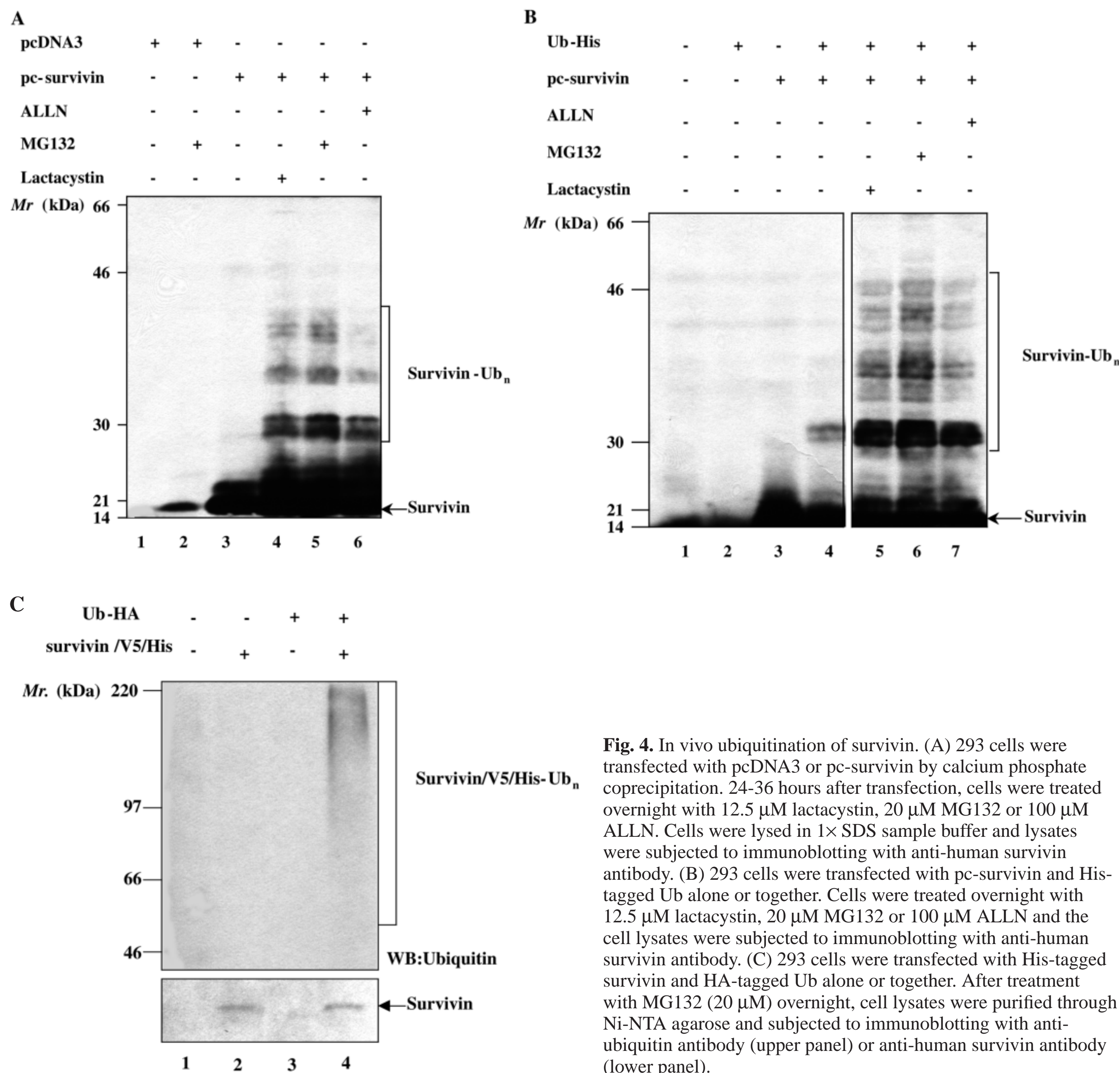

Fig. 4. In vivo ubiquitination of survivin. (A) 293 cells were transfected with pcDNA3 or pc-survivin by calcium phosphate coprecipitation. 24-36 hours after transfection, cells were treated overnight with 12.5 μM lactacystin, 20 μM MG132 or 100 μM ALLN. Cells were lysed in 1× SDS sample buffer and lysates were subjected to immunoblotting with anti-human survivin antibody. (B) 293 cells were transfected with pc-survivin and His-tagged Ub alone or together. Cells were treated overnight with 12.5 μM lactacystin, 20 μM MG132 or 100 μM ALLN and the cell lysates were subjected to immunoblotting with anti-human survivin antibody. (C) 293 cells were transfected with His-tagged survivin and HA-tagged Ub alone or together. After treatment with MG132 (20 μM) overnight, cell lysates were purified through Ni-NTA agarose and subjected to immunoblotting with anti-ubiquitin antibody (upper panel) or anti-human survivin antibody (lower panel).

inhibitors supports our conclusion that proteasomes are involved in survivin degradation.

Secondly, we demonstrated ubiquitination of survivin in vivo, by cotransfection of Ub-expressing plasmids or inhibition of proteasome function with MG132. While ubiquitination might result from blocking degradation, we provide direct evidence of ubiquitination of survivin by comparing coexpression of survivin with either wild-type ubiquitin or the K48R ubiquitin mutant. The K48R mutant has a dominant effect on polyubiquitination and degradation, and causes production of more stable ubiquitinated products than wild-type ubiquitin (Ward et al., 1995). In addition, after nickel affinity chromatography, polyubiquitinated survivin was only detected in lysates of cells coexpressing both His-tagged survivin and ubiquitin. These in vivo ubiquitination data support our conclusion that polyubiquitination is a signal for survivin degradation.

Thirdly, survivin expression and ubiquitination is regulated in a cell cycle-dependent manner. Survivin levels were shown to be cell cycle-dependent with a decline in G_1 phase and an increase in G_2/M phase. The demonstration that the G_1 phase decline was blocked by proteasome inhibitors supports the hypothesis that survivin is degraded by the proteasome-ubiquitin pathway. To demonstrate further that survivin degradation is cell cycle-regulated we expressed survivin under control of the CMV promoter, which is not regulated by the cell cycle. If survivin was regulated only at the transcriptional level we would expect to see no change in protein level when

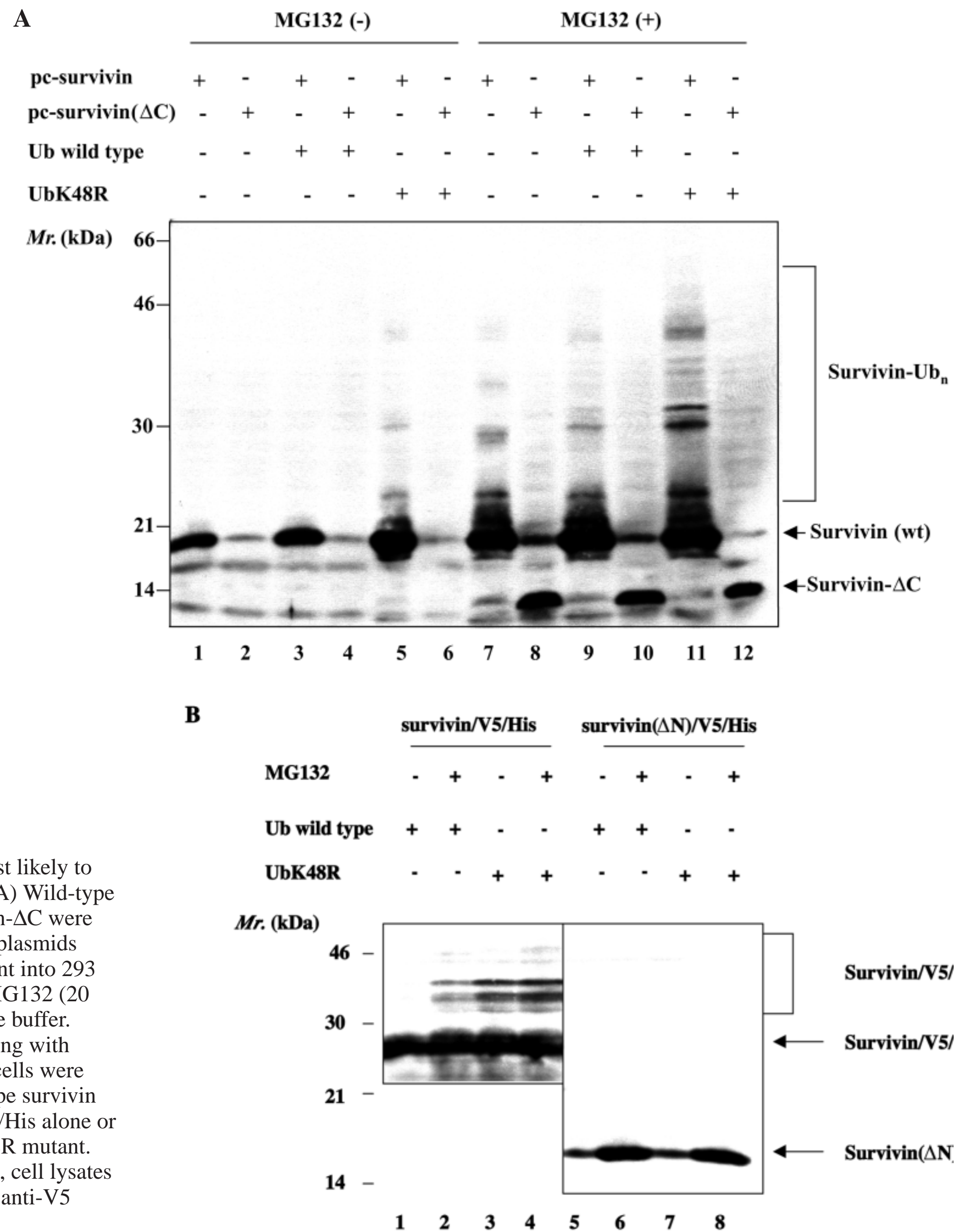

Fig. 5. Ubiquitination of survivin is most likely to occur through several lysine residues. (A) Wild-type survivin and truncated survivin, survivin-ΔC were transfected alone or cotransfected with plasmids expressing wild-type Ub or K48R mutant into 293 cells. After treatment with or without MG132 (20 μM), cells were lysed in 1× SDS sample buffer. Lysates were subjected to immunoblotting with anti-human survivin antibody. (B) 293 cells were transfected with His/V5-tagged wild-type survivin and truncated survivin, survivin-ΔN/V5/His alone or cotransfected with wild-type Ub or K48R mutant. After treatment with or without MG132, cell lysates were subjected to immunoblotting with anti-V5 antibody.

survivin is expressed from the CMV promoter. In contrast we found a decline in the level of survivin at G_1, which suggests a post-transcriptional, cell cycle-dependent mechanism of degradation.

Fourthly, using various survivin-lysine mutants (data not shown) and the K48R ubiquitin mutant, we were able to demonstrate that survivin is ubiquitinated on several lysine residues. Cotransfection of the K48R mutant with survivin produced higher levels of polyubiquitination products, which are the result of mono-K48RUb conjugates on multiple lysine residues.

Finally, we demonstrated that deletion mutants or point mutations in critical amino acid residues within the BIR domain greatly sensitise survivin to ubiquitin-proteasome degradation. Transient overexpression of the C84A mutant has been described previously to have a dominant negative effect on endogenous survivin (Li et al., 1998). This mutant has been used for targeting of cancer cells (Grossman et al., 1999a; Grossman et al., 1999b; Li et al., 1998). However, our results suggest that the ubiquitin pathway can interfere with the stability of these mutants. The C84A mutant was not able to induce apoptosis as has been reported in some other systems (Grossman et al., 1999a; Grossman et al., 1999b; Li et al., 1998), which may be explained by its rapid degradation through the proteasome (data not shown). Generation of a C84A mutant 'resistant' to ubiquitin degradation could have a more potent effect in cancer cells. Regulation of survivin ubiquitination still remains to be fully understood. It is unlikely to be related to the binding of microtubules, since disruption of microtubules by cytochalasin D and nocodazole (data not

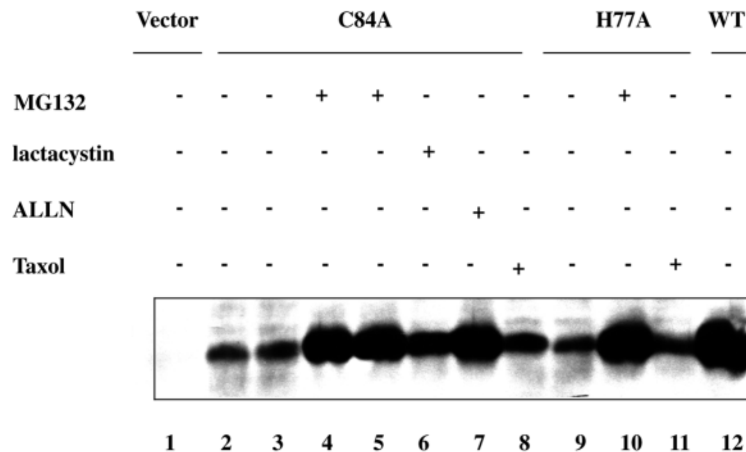

Fig. 6. Point mutations within the BIR domain sensitise survivin to ubiquitin-proteasome degradation. Wild-type survivin or survivin mutants C84A and H77A were transfected into 293 cells. After treatment with proteasome inhibitors or 1 μM Taxol, cells were lysed in 1% Triton buffer and subjected to immunoblotting with anti-human survivin antibody.

shown) did not enhance degradation of survivin. Nor is it likely to be related to the location of survivin. The tail-less (C-terminally truncated) mutant survivin, which fails to associate with microtubules and accumulates in the cytoplasm (Li et al., 1998), was still degraded by the proteasome. Further comparison between the truncation mutants revealed that the tail-less mutant was even more sensitive to proteasome degradation then the mutant truncation at the N terminus. This could be explained by an involvement of the N-terminal part of survivin in interaction with the E3 ubiquitin ligases.

Although we have shown that the ubiquitin-proteasome pathway is crucial for survivin degradation, the specific enzymes involved in this process have yet to be determined, especially the E3 ubiquitin ligases which are responsible for substrate recognition by the ubiquitin machinery.

Deregulation of survivin has been reported at both RNA and protein levels, with survivin protein accumulating in the cytoplasm (Ambrosini et al., 1997; Kawasaki et al., 1998; LaCasse et al., 1998; Lu et al., 1998). Defects in the ubiquitin-proteasome pathway have been linked to different cancers and genetic diseases (Ciechanover, 1998). Dysfunction of the ubiquitin-proteasome pathway might contribute to the deregulation of survivin in cancer cells. The results presented here not only improve our understanding of the regulation of survivin during the cell cycle, but also provide important information to guide the design of survivin mutants for anti-cancer therapy.

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