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Role of proteasomal degradation in the cell cycle-dependent regulation of DNA topoisomerase IIα expression☆

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Abstract

DNA topoisomerase II (topo II) is a nuclear enzyme that modifies DNA topology and also serves as a target to mediate the cytotoxicity of several antineoplastic agents. Several reports have demonstrated that a reduction of topo II is associated with reduced sensitivity to these agents. Topo II exists as two isoforms in mammalian cells: topo IIα and topo IIβ. In MCF-7 cells, the half-life (mean ± SEM) values of topo IIα and topo IIβ *in situ* were 6.6 ± 0.3 and 17.6 ± 2.3 hr, respectively, as determined by [^{35}S]methionine/cysteine pulse-chase analysis. Degradation of topo IIα *in situ* was abrogated by the presence of proteasome inhibitors, and the relative activities were carbobenzoxy-leucyl-leucyl-leucinal (MG132) > carbobenzoxy-leucyl-leucyl-norvalinal (MG115) > ALLN ≅ lactacystin. ATP-dependent degradation of topo IIα, but not topo IIβ, was observed in extracts of asynchronously dividing HeLa and MCF-7 cells. Furthermore, degradation of topo IIα was abrogated by the proteasome inhibitors MG132 and MG115, but not by lactacystin, in extracts of asynchronously dividing MCF-7 cells. Finally, degradation of topo IIα, but not topo IIβ, was observed to occur in a cell cycle-dependent fashion, in extracts of synchronized HeLa cells, with maximal loss of the α isoform occurring 2 hr after release from mitotic arrest. This degradation of topo IIα appeared to be facilitated by an ATP-dependent activity. Furthermore, high molecular weight bands (>200 kDa), which may represent polyubiquitinated–topo IIα conjugates, were also detected in extracts of synchronized HeLa cells. This study provides evidence for a role of the ubiquitin–proteasome pathway in the cell cycle-dependent regulation of topo IIα expression. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Topoisomerase IIα; Proteasome; Degradation; Ubiquitin; MG132; Cell cycle

1. Introduction

DNA topo II is a nuclear enzyme that regulates DNA topology and also serves as a target for several antineoplastic agents [1]. The reduction of topo II has been implicated as a mechanism of resistance to anti-cancer drugs [2, 3]. Two isoforms of topo II have been identified, topo IIα and topo IIβ, which have distinct patterns of expression during the cell cycle [4]. The α-isoform is expressed in a cell cycle-dependent manner with levels that are low during G_1, rise during S, and peak during G_2 and mitosis [5–8]. By contrast, expression of topo IIβ is relatively constant throughout the cell cycle [5]. The half-life ($T_{1/2}$) of topo IIα in synchronously dividing cells is 7-fold lower during the first 2 hours of G_1, compared with the $T_{1/2}$ in asynchronously dividing cells [5]. Furthermore, it was reported that 35% of topo IIα is degraded within 4 hr of the transition from M to G_1 [9]. Recent studies have shown that topo IIα is a target for ubiquitin-mediated proteolysis [10, 11]. Ubiquitin-mediated proteolysis involves the ubiquitination of a target protein that is subsequently degraded by the 26S proteasome [12]. This study was undertaken to test the hypothesis that expression of topo IIα is cell cycle-dependent and regulated by proteasome-mediated degradation.

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Abbreviations: topo II, DNA topoisomerase II; MG132, carbobenzoxy-leucyl-leucyl-leucinal; MG115, carbobenzoxy-leucyl-leucyl-norvalinal; ALLN, acetyl-leucyl-leucyl-norleucinal; α-MEM, α-Minimum Essential Medium; and D-MEM, Dulbecco's Minimum Essential Medium.

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2. Materials and methods

2.1. Cell culture and synchronization protocols

HeLa and MCF-7 cell lines were maintained in exponential phase in α-MEM supplemented with 1 mg/mL of penicillin G and streptomycin sulfate and 10% fetal bovine serum (Cansera International), at 37° and 5% CO_2. HeLa cells were synchronized at mitosis by exposure to aphidicolin and nocodazole, using minor modifications of methods described previously [6, 13]. Approximately 2×10^6 HeLa cells in exponential growth were incubated for 16 hr in medium containing 2 μg/mL of aphidicolin, washed three times in serum-free medium, and incubated for 8 hr in α-MEM containing 0.05 μg/mL of nocodazole. Cells were collected using a rubber policeman, washed three times in serum-free medium, replated in α-MEM, and harvested at 0, 1, 2, and 4 hr after release from nocodazole block.

2.2. Pulse-chase analysis of topo II degradation in vivo

MCF-7 cells were incubated in D-MEM without methionine and cysteine (ICN Biochemicals) for 15 min to deplete intracellular pools of those amino acids. Cellular proteins were radiolabeled by incubating cells for 10 hr in methionine- and cysteine-free D-MEM containing 0.1 mCi/mL of [^{35}S]methionine/cysteine (Tran [35]S-label, ICN Radiochemicals) and 10% normal D-MEM.

Radiolabeled proteins were chased by incubation in D-MEM containing 1 mM methionine and cysteine. In studies of the effects of proteasome inhibitors, MG115, MG132, ALLN (Calbiochem), and lactacystin were added to the chase medium. Cells were either collected and stored at −70° or lysed immediately in lysis buffer. Topo IIα and topo IIβ were immunoprecipitated from 200 μg of whole cell protein with a 1:10 dilution of monoclonal antibody 8D2 or 5A7, respectively. Immunoprecipitates were washed three times in lysis buffer, before loading onto a 7.5% SDS-polyacrylamide gel. The gels were fixed and dried under vacuum for 1 hr at 80° and exposed to a Phosphor screen for 48 hr. Radiolabeled topo II was visualized with a Storm 860 Imaging System, and quantitation was performed using ImageQuant analysis software.

2.3. Immunoprecipitation of topo IIα and topo IIβ

Approximately 1×10^8 cells in log phase were washed twice in ice-cold PBS and lysed for 30 min in buffer [50 mM HEPES (pH 7.0), 1 mM EDTA, 420 mM NaCl, 0.1% Nonidet P-40] containing protease inhibitors (174 μg/mL of phenylmethylsulfonyl fluoride, 20 μg/mL of aprotinin, 25 μg/mL of leupeptin, 1 μg/mL of pepstatin, 1 μg/mL of antipain, 157 μg/mL of benzamidine, 1 μg/mL of trypsin) on ice. The lysate was centrifuged at 12,000 *g* for 10 min at 4° to remove cellular debris. The supernatant was precleared with 200 μL of protein A-Sepharose beads 6 MB (Pharmacia Biotech) for 1 hr at 4°. Lysates were incubated with 400 μL of anti-human topo II antibodies 8D2 or 5A7 for 1 hr at 4° and were immunoprecipitated with 500 μL of protein A-Sepharose beads overnight at 4°. After centrifugation at 10,000 *g*, the immunoprecipitates were washed twice in lysis buffer containing protease inhibitors and twice in buffer without protease inhibitors. Immunoprecipitates were stored at 4° as a 10% suspension in hypotonic buffer.

2.4. Preparation of S10 crude cell homogenates

Approximately 2×10^8 exponentially growing cells were harvested, washed twice in 5 mL PBS and once in 5 mL hypotonic buffer [20 mM Tris (pH 7.4), 5 mM MgCl, 8 mM KCl, 1 mM dithiothreitol], resuspended in 2 mL of the same buffer, and incubated on ice for 15 min. The swollen cells were collected in a Dounce homogenizer and centrifuged at 300 *g* for 5 min at 4°. The excess buffer was aspirated, and the cells were disrupted with 60 strokes of a tight-fitting pestle. The homogenate was centrifuged twice at 10,000 *g* for 5 min at 4°, and the turbid supernatant, which constitutes the S10 extract, was collected. The protein concentration was determined [14], and the S10 extract was stored at −70°.

2.5. Degradation of topo IIα

Degradation of topo IIα was determined on S10 crude cell homogenates using minor modifications of methods described previously [11]. ATP and ubiquitin were added alone or together as 2 mM ATP (Pharmacia, Biotech) with an ATP-regenerating system consisting of 10 mM creatine phosphate and 25 U/mL of creatine phosphokinase and/or 0.1 μg/μL of purified ubiquitin (Sigma). Proteasome inhibitors MG132 and MG115 (Peptide Institute Inc.) and lactacystin (purchased from Dr. E. J. Corey, Harvard University) were added at the indicated concentrations. Immunoprecipitated topo II served as the substrate; the reaction was incubated at 37° for 90 min and stopped by cooling on ice for 5 min and centrifugation at 8000 *g* for 2 min at 4°. The immunoprecipitates were washed four times in lysis buffer and three times in distilled H_2O, resuspended in loading buffer [125 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 1.43 M β-mercaptoethanol], and boiled for 5 min to extract protein from the beads.

2.6. DNA staining with propidium iodide

DNA was stained and analyzed by flow cytometry to determine the adequacy of the blocking protocols. Approximately 5×10^6 cells were harvested, washed twice in ice-cold PBS, fixed in 70% ethanol, and stored overnight at −20°. Cells were rehydrated in ice-cold PBS, rinsed once in PBS containing 0.12 mM EDTA and 0.12% Triton X-100 (Sigma), and incubated in 250 μg/mL of RNase A (Sigma) for 30 min at 37°. Propidium iodide (Sigma) was added to

the cell suspensions at a final concentration of 5 μg/mL. Flow cytometry was performed with a FACSCalibur flow cytometer (Becton Dickinson) recording at least 10,000 events using ModFit LT analysis software (Verity Software House Inc.).

2.7. Western blotting

Immunoprecipitated proteins were resolved on 7.5% polyacrylamide gels, run at 200 mV for 1 hr, and transferred onto PVDF membranes (Immobilon P, Millipore) using a wet transfer system (Bio-Rad). Membranes were blocked in 5% non-fat dry milk in TST (20 mM Tris, 136.9 mM NaCl, 0.1% Tween-20) for 1 hr at room temperature and incubated overnight at 4° with a 1:50 dilution of monoclonal anti-topo IIα or anti-topo IIβ antibodies. Immunodetection was performed using the Vistra^{TM} Fluorescence Western Blotting kit (ECF, Amersham). Fluorescence was detected with a Storm 860 Imaging System (Molecular Dynamics), and quantitation was performed using ImageQuant analysis software (Molecular Dynamics). Monoclonal mouse antibodies 8D2 and 5A7 raised against human topo IIα and topo IIβ, respectively, were provided by Dr. Akihiko Kikuchi [11, 13].

3. Results

3.1. Decay-time analysis of topo IIα and topo IIβ in MCF-7 cells in vivo

The half-lives ($T_{1/2}$) of topo IIα and topo IIβ in MCF-7 cells *in vivo* were determined by [^{35}S]methionine/cysteine pulse-chase analysis (Fig. 1A). A decay-time analysis of three independent experiments showed that the $T_{1/2}$ (mean ± SEM) values for topo IIα and topo IIβ were 6.6 ± 0.3 and 17.6 ± 2.3 hr, respectively, and this 2.7-fold difference was highly significant ($P < 0.0005$). These findings suggest that the regulation of topo II expression in MCF-7 cells is isoform-dependent, and that the α-isoform is significantly less stable than the β-isoform.

The effect of the proteasome inhibitor MG132 on the $T_{1/2}$ of topo IIα was examined in MCF-7 cells (Fig. 1B). As shown in the pulse-chase analysis, MG132 inhibited the degradation of topo IIα. The $T_{1/2}$ of topo IIα in MCF-7 cells in the absence of MG132 was 9.8 hr, whereas that in the presence of 10 μM MG132 was 59.3 hr; this 6-fold increase was highly significant ($P < 0.0005$).

3.2. Effect of proteasome inhibitors MG132, MG115, lactacystin, and ALLN on the degradation of topo IIα in MCF-7 cells in vivo

The effects of four proteasome inhibitors on the degradation of topo IIα in MCF-7 cells *in vivo* were determined (Fig. 1C). The level of topo IIα in untreated control cells after a 24-hr pulse chase decreased to 24.9 ± 5.5% (Fig. 1C; left panel) or 28.9 ± 9.9% (Fig. 1C; right panel) of the initial level, and these differences were statistically significant. The degradation of topo IIα was blocked by the presence of MG132, MG115, or 10 μM lactacystin, and in each case the effect was statistically significant; the abrogation observed with 1 μM lactacystin or ALLN fell short of statistical significance. In this study, the natural decay of radiolabeled topo IIα was blocked in a concentration-dependent manner by the proteasome inhibitors, and the relative activity was MG132 > MG115 > ALLN ≅ lactacystin.

3.3. ATP-dependent degradation of topo IIα in cell-free extracts of MCF-7 and HeLa cells

Degradation of topo IIα was investigated using extracts of asynchronously dividing HeLa and MCF-7 cells (Fig. 2). The level of topo IIα in HeLa and MCF-7 cell extracts was unaltered by the addition of ubiquitin. However, the level of topo IIα (mean ± SEM) following the addition of 2 mM ATP to HeLa cell extracts was 15.3 ± 6.8% of that of untreated controls, and the difference was statistically significant ($P = 0.012$, paired *t*-test). The level of topo IIα in MCF-7 cell extracts following ATP dropped to 36.1 ± 3.3% of untreated controls, and this difference was also significant ($P = 0.016$). The addition of both ATP and ubiquitin to extracts of HeLa and MCF-7 cells reduced topo IIα levels to 17.0 ± 5.7 and 24.4 ± 6.3% of controls, respectively, and these changes were significant ($P = 0.023$ and 0.042, respectively). These findings suggest that degradation of topo IIα in cell-free extracts of HeLa and MCF-7 cells is ATP-dependent.

3.4. Effect of proteasome inhibitors on the degradation of topo IIα in cell-free extracts of MCF-7 cells

To investigate the possible involvement of the ubiquitin–proteasome pathway, the effects of three proteasome inhibitors on the degradation of topo IIα were examined (Fig. 3). Degradation of topo IIα was determined in cell-free extracts of MCF-7 cells in the absence and presence of MG132, MG115, and lactacystin. In the presence of ATP and ubiquitin, topo IIα levels decreased to levels between 33 and 48% of that observed in untreated controls. In the presence of MG132 and MG115, a concentration-dependent increase in topo IIα was observed, suggesting that degradation of topo IIα was abrogated by these proteasome inhibitors; however, the difference fell short of statistical significance, as determined by analysis of variance. No abrogation was observed with lactacystin. These findings suggest that topo IIα may be degraded by a proteasome-mediated process.

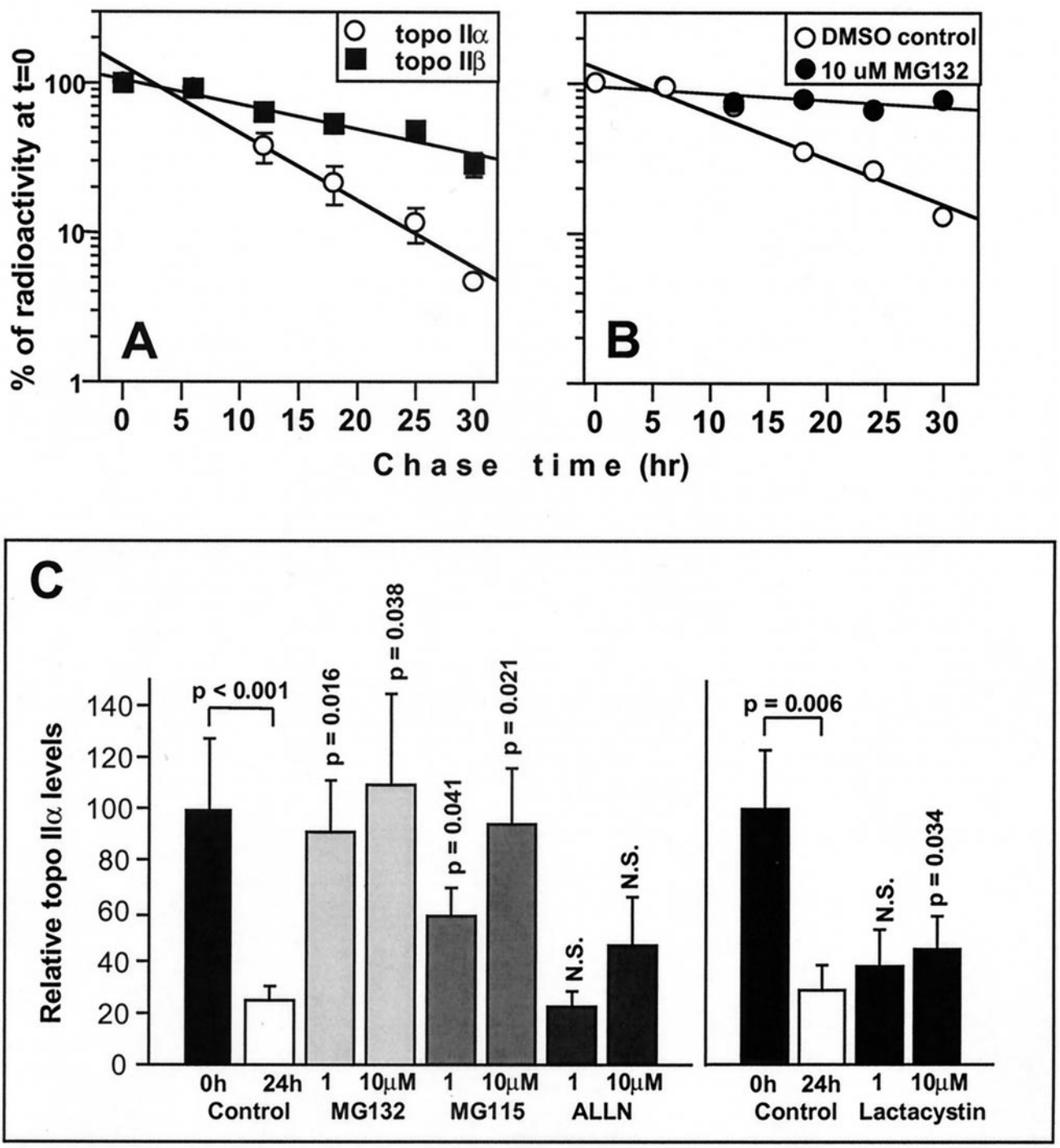

Fig. 1. Decay-time analysis of topo IIα and topo IIβ in MCF-7 cells *in vivo*. (A) The $T_{1/2}$ values of topo IIα and topo IIβ in MCF-7 cells were determined in cells that were labeled with [^{35}S]methionine/cysteine for 10 hr, and the radioactivity was chased for 24 hr in medium containing an excess of unlabeled methionine and cysteine. The $T_{1/2}$ (mean $\pm$ SEM) values of topo IIα and topo IIβ were 6.6 $\pm$ 0.3 and 17.6 $\pm$ 2.3 hr, respectively, and this difference was statistically significant ($P < 0.0005$) as determined by a *t*-test comparing the significance of the difference of slopes. Data points represent the means $\pm$ SEM of 3 independent experiments. (B) Pulse-chase analysis of topo IIα in MCF-7 cells. The $T_{1/2}$ of topo IIα in untreated control cells was 9.8 hr and that of cells exposed to 10 μM MG132 was 59.3 hr; this 6-fold difference was statistically significant ($P < 0.0005$, *t*-test for the significance of the difference of slopes). (C) The effects of MG132, MG115, ALLN, and lactacystin on the degradation of topo IIα in MCF-7 cells were measured. Cells were radiolabeled for 10 hr, and the label was chased for 24 hr in the absence or presence of proteasome inhibitors. Data represent the means $\pm$ SEM of 3 independent experiments.

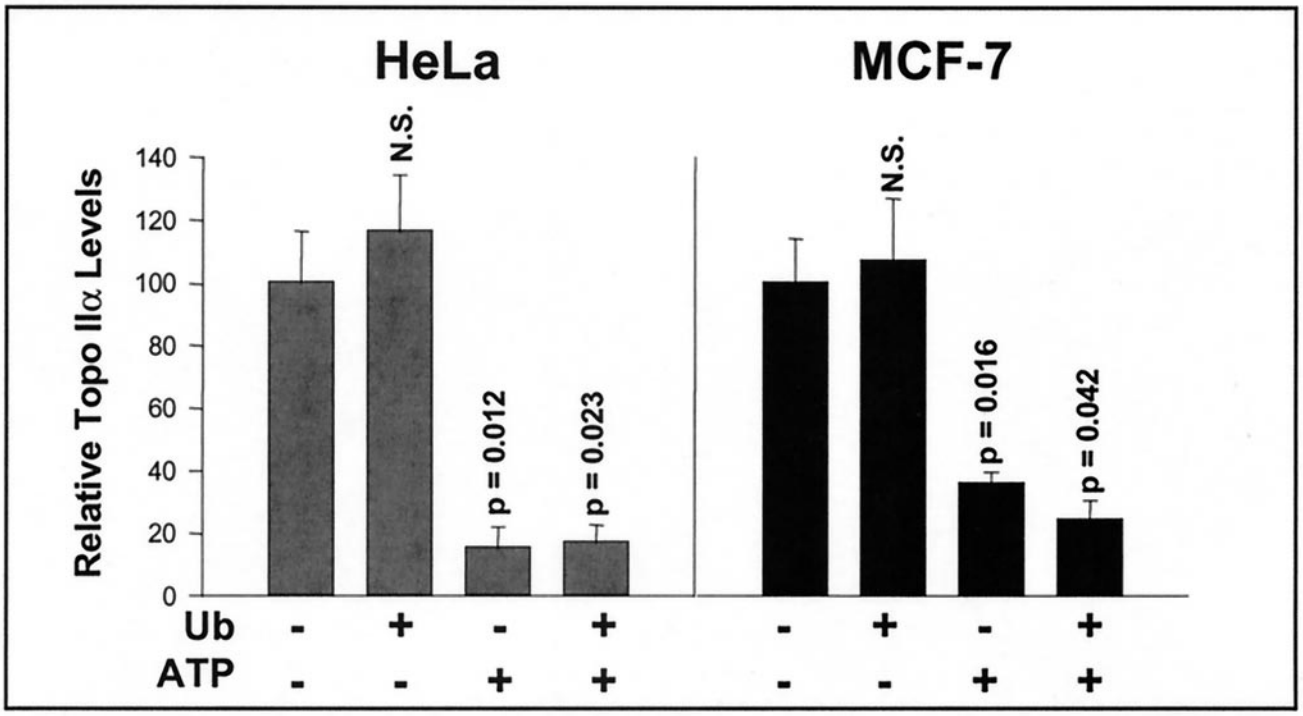

Fig. 2. Degradation of topo IIα in extracts of asynchronous HeLa and MCF-7 cells. Immunoprecipitated topo IIα was incubated with S10 extracts from HeLa and MCF-7 cells for 1.5 hr at 37° in the presence or absence of ATP and ubiquitin. Data represent the means ± SEM of 3 independent experiments.

3.5. Degradation of topo IIα in extracts of synchronized HeLa cells

A study of topo IIα degradation was undertaken in cell-free extracts of HeLa cells that had been synchronized by sequential exposure to aphidicolin and nocodazole (Fig. 4). DNA histograms of HeLa cells arrested at mitosis and up to 4 hr after mitotic arrest are shown in Fig. 4A. The addition of ATP to cell-free extracts prepared from these synchronized cells resulted in a reduction of topo IIα levels, whereas the addition of ubiquitin showed little effect (Fig. 4B). These results suggest that the degradation of topo IIα in extracts of synchronized HeLa cells is ATP-dependent.

The degradation of topo IIβ was also examined in these

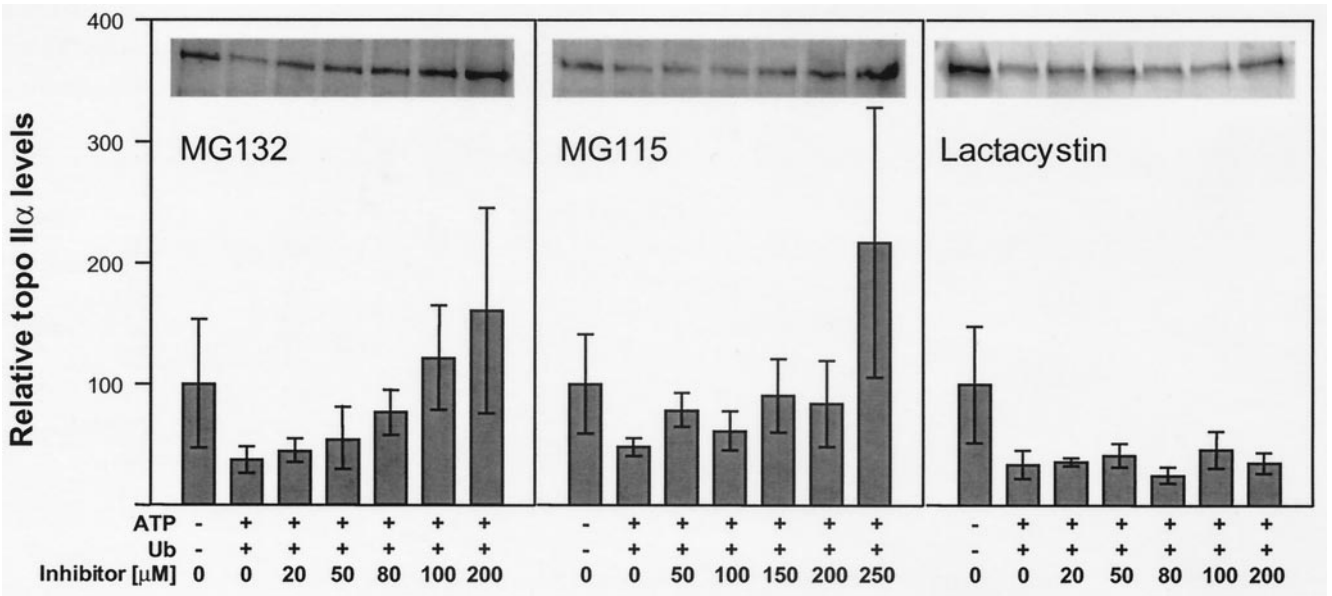

Fig. 3. Effects of the proteasome inhibitors MG132, MG115, and lactacystin upon degradation of topo IIα in cell-free extracts of MCF-7 cells. Data represent the means ± SEM of 3 independent experiments. Representative Western blots are displayed.

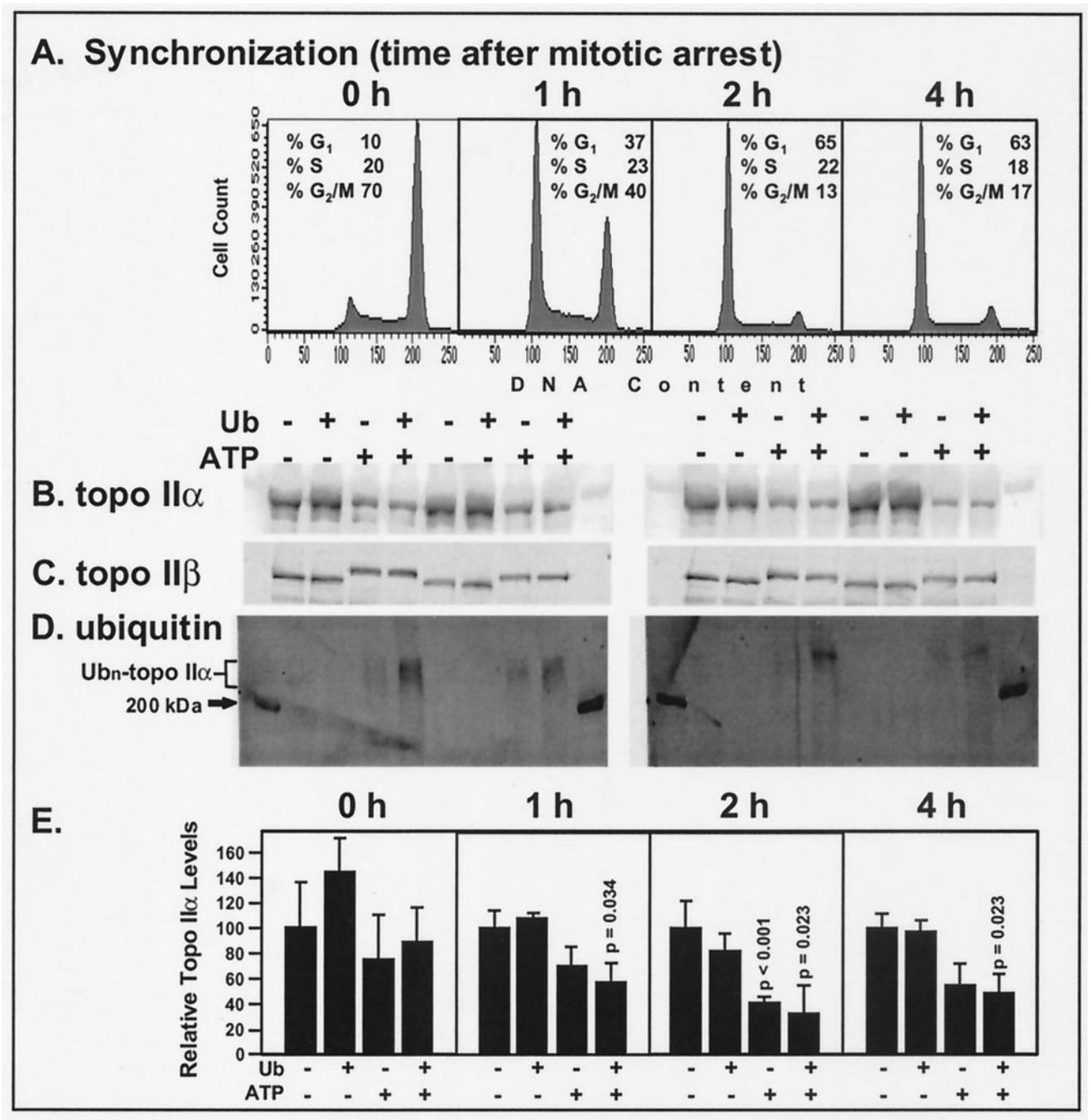

Fig. 4. Degradation of topo IIα in extracts of synchronized HeLa cells. (A) The degradation and ubiquitination of topo IIα and topo IIβ were examined in extracts of synchronized HeLa cells at 0, 1, 2, and 4 hr after mitosis. (B) Topo IIα immunoprecipitates were incubated with cell-free extracts for 1.5 hr at 37° and subsequently analyzed by Western blotting. Monoclonal anti-topo IIα (8D2) antibody was used for the immunodetection of topo IIα levels. (C) Topo IIβ immunoprecipitates were subjected to analogous conditions, and immunodetection was performed with monoclonal anti-topo IIβ (5A7) antibody. (D) Topo IIα Western blots (as shown in panel B) were stripped and reprobed with a polyclonal anti-ubiquitin antibody to detect topo IIα–ubiquitin conjugates. Dark bands in the flanking lanes of the Western blots represent a 200 kDa molecular weight marker. (E) Quantitation of topo IIα upon the addition of ubiquitin. Data represent the means ± SEM of 4 independent experiments.

extracts (Fig. 4C). There was no reduction of topo IIβ in extracts treated with ATP and/or ubiquitin, suggesting that the β-isoform is not degraded under these conditions. However, a reduction in the mobility of topo IIβ was observed in the presence of ATP or ATP and ubiquitin, suggesting an increase in molecular weight of that isoform, which may represent phosphorylation of the β-isoform during mitosis, as reported by others [9, 13]. Further studies to elucidate the nature of this alteration were not undertaken. These findings demonstrated that topo IIβ is not degraded, suggesting that degradation of topo IIα is specific and not a general phenomenon occurring with proteins in this cell-free system.

The demonstration of topo IIα–ubiquitin conjugates would constitute strong evidence that degradation of topo IIα is ubiquitin-mediated. To investigate the formation of conjugates, membranes initially probed for topo IIα (Fig. 4B) were stripped and reprobed with an anti-ubiquitin antibody (Fig. 4D). Bands of high molecular weight (>200 kDa), which may represent polyubiquitinated–topo IIα conjugates, were detected in extracts treated with ATP and ubiquitin at 0, 1, and 2 hr, and to a lesser extent at 4 hr, after synchronization. A faint band was also observed in extracts treated with ATP alone at 0 and 1 hr after mitotic arrest. However, the bands detected by the anti-ubiquitin antibody did not cross-react with the anti-topo IIα antibody.

A series of four independent studies of topo IIα degradation were undertaken in extracts of synchronized HeLa cells, as shown in Fig. 4E. There was no change in the level of topo IIα upon the addition of ubiquitin to these extracts. There was a decrease in the level of topo IIα upon the addition of ATP, but this reached significance only in extracts from cells taken 2 hr after synchronization ($P < 0.001$). The level of topo IIα was also reduced by the addition of ATP and ubiquitin, and this reached significance for cell extracts obtained 1, 2, and 4 hr after mitotic arrest ($P = 0.034$ or greater). These findings suggest that topo IIα degradation in extracts of synchronized cells is ATP- and ubiquitin-dependent, with the most profound effect occurring 2 hr after mitotic arrest.

4. Discussion

A decay-time analysis of topo IIα and β in MCF-7 cells *in vivo* revealed that the turnover of topo IIα was 2.7-fold more rapid than that of the β isoform (Fig. 1A). Furthermore, the $T_{1/2}$ of topo IIα was increased 6-fold in the presence of the proteasome inhibitor MG132 (Fig. 1B). In addition to MG132, degradation of topo IIα *in vivo* was also abrogated by the presence of the proteasome inhibitors MG115, ALLN, and lactacystin (Fig. 1C), and the relative activities were MG132 > MG115 > ALLN ≅ lactacystin. These findings provide evidence that degradation of topo IIα in MCF-7 cells may be proteasome-mediated.

In this study, a cell-free system was used to study degradation and ubiquitination of topo IIα *in vitro*. Under these experimental conditions, synthesis of topo IIα or β would not be expected to occur so any reduction in the level of either isoform could be attributed to degradation. Reduction in the level of topo IIα in extracts of asynchronous HeLa and MCF-7 cells was demonstrated to be ATP-dependent (Fig. 2). The observation that degradation was ATP-dependent is consistent with the involvement of the ubiquitin–proteasome pathway.

In synchronized HeLa cells, evidence was also found of degradation of topo IIα, but not topo IIβ, and the degradation was ATP- and ubiquitin-dependent (Fig. 4). The detection of high molecular weight bands (>200 kDa) by the anti-ubiquitin antibody in extracts of HeLa cells synchronized at mitosis and early G_1 suggested the formation of topo IIα–ubiquitin conjugates (Fig. 4D). These bands did not cross-react with the anti-topo IIα antibody, and there are at least two possible explanations for this finding. First, the anti-ubiquitin antibody may be detecting ubiquitin conjugates other than topo IIα. Second, extensive ubiquitination of topo IIα may mask the epitope sites recognized by the anti-topo IIα antibody [15].

Degradation of topo IIα in extracts of asynchronous MCF-7 cells was abrogated, in a concentration-dependent manner, by the tripeptide proteasome inhibitors MG132 and MG115 (Fig. 3). This finding supports the notion that the proteasome is involved in mediating the cell-free degradation of topo IIα. However, no abrogation of topo IIα degradation was observed with lactacystin.

The relative activity of the three proteasome inhibitors MG132, MG115, and ALLN was consistent with previous reports in the literature [16, 17]. However, the finding of the relatively low potency of lactacystin differs with reports that it is the most potent and specific proteasome inhibitor [18, 19]. One possible explanation for the relatively low activity of lactacystin is that it does not enter cells readily, but must be converted by cyclization to the *clasto*-lactacystin β-lactone that does readily enter cells. The demonstration of activity of lactacystin *in vivo* contrasts with the absence of activity *in vitro* and may be attributable to the concentrative accumulation of the β-lactone by intact cells [18]. Another possibility is that proteolytic pathways other than proteasome-mediated degradation of topo IIα may be involved.

The tripeptide inhibitors used in this study have been reported to inhibit calpain-mediated proteolysis [17]. Calpains, a family of cytosolic cysteine proteases, are activated by controlled increases in intracellular calcium [20]. Calpain inhibitor I, also known as ALLN and MG101, is also an effective proteasome inhibitor. To clarify the role of calpains in the degradation of topo IIα, it would be useful to study the effect of more specific calpain inhibitors such as calpain inhibitor II and calpastatin.

Although degradation of topo IIα is postulated to occur primarily during the G_1 phase of the cell cycle, the mechanism responsible for this regulation is unknown. In studying possible mechanisms of degradation, the expression of topo IIα may be compared with that of other proteins such as cyclin B, p55Cdc, and NIMA protein kinase, which are regulated in a cell cycle-dependent manner mediated, at least in part, by ubiquitin-dependent proteolysis [21–24]. The isoform specificity and the rapid reduction of topo IIα during early G_1 suggest that degradation of topo IIα, but not topo IIβ, may also be mediated by ubiquitin-dependent proteolysis.

Understanding the mechanism whereby topo IIα is elevated in the presence of proteasome inhibitors (Fig. 1, B and C) might be complicated by the effect of the inhibitors on cell cycle distribution in that cells could be arrested in G_2/M at which phase topo IIα levels peak [5–8]. Therefore, we

examined the effect of MG132 on cell cycle distribution of asynchronously dividing MCF-7 cells. The percentage of G_2/M cells in the absence of the proteasome inhibitor varied from 6 to 12%, whereas that in the presence of 10 μM MG132 for 24 hr ranged from 14 to 20%, so that there was approximately a 2-fold increase in the percentage of G_2/M cells. In this study, the level of topo IIα (mean $\pm$ SEM) in the absence of inhibitor was 24.9 $\pm$ 5.5% of control, that in the presence of 10 μM MG132 was 109.1 $\pm$ 35.4%, and this greater than 4-fold increase was highly significant (Fig. 1C). It is not possible from this study to determine how much of the increase in topo IIα in the presence of proteasome inhibitors was due to G_2/M arrest compared with inhibition of proteasomal degradation. However, given the magnitude of the increase in topo IIα levels compared with the increase of cells in G_2/M, it would appear that the increase of topo IIα, at least in part, is mediated by inhibition of proteasomal degradation.

Our findings are consistent with several recent reports in the literature [10, 11, 25, 26]. Nakajima *et al.* [10, 11] demonstrated ubiquitin–topo IIα conjugates in MA1 epidermoid carcinoma cells and suggested that topo IIα degradation during adenovirus E1A-induced apoptosis is mediated by the ubiquitin proteolysis system. The Tsuruo group reported that topo IIα expression was down-regulated at the G_1-phase by proteasome-mediated degradation in glucose-stressed cancer cells [25], and that proteasome inhibition attenuated resistance to topo II-interactive agents by restoring levels of topo IIα [26]. Our findings provide evidence for the regulation of topo IIα by this degradation pathway through the normal cell cycle. Proteasomal degradation of topo IIα may represent a mechanism whereby cells become resistant to topo II-interactive antineoplastic agents and provides a rationale for the use of proteasome inhibitors to circumvent resistance to these drugs.

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