学　位　論　文

Involvement of pRB-Related p107 Protein in the Inhibition of S-Phase Progression in Response to Genotoxic Stress

DNA 傷害性ストレスに対するレチノブラストーマ癌抑制遺伝子産物（pRB）相同分子・p107 による DNA 合成期進行の抑制について

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Involvement of pRB-Related p107 Protein in the Inhibition of S-Phase Progression in Response to Genotoxic Stress

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SUMMARY

pRB-family pocket proteins consisting of pRB, p107, and p130 are thought to act as a set of growth regulators that inhibit the cell-cycle transition from G1- to S-phases by virtue of their interaction with E2F transcription factors. When cells are committed to progressing through the cell-cycle at the late G1 restriction point, they are hyperphosphorylated by G1 cyclin-cyclin dependent kinase (CDK) and are functionally inactivated. Consistent with such a G1 regulatory role, pRB and p130 are abundantly expressed in quiescent cells. In contrast, p107 is present at low levels in the hypophosphorylated form in quiescent cells. As cells progress toward late G1- to S-phases, the levels of p107 increase and the majority become hyperphosphorylated, suggesting a possible role of p107 in post-G1 cell-cycle regulation. In this study, we have demonstrated that a non-phosphorylatable and thus constitutively active p107 has the potential to inhibit S-phase progression. The levels of the phosphorylation-resistant p107 required for the S-phase inhibition are significantly less than those of endogenous p107. We further show herein that the exposure of cells to the DNA-damaging agent, cisplatin, provokes S-phase arrest, which is concomitantly associated with the accumulation of hypophosphorylated p107. Furthermore, the S-phase inhibitory response to cisplatin is augmented by the ectopic expression of wild-type p107, while it is diminished by the adenovirus E1A oncoprotein, which counteracts the pocket protein functions. Because p107 is a major pRB-family protein expressed in S-phase cells, our results indicate that p107 participates in an inhibition of cell-cycle progression in response to DNA damage in S-phase cells.

INTRODUCTION

The decision to commit to cell division takes place at a late G1 point, termed the restriction point, that precedes the onset of DNA synthesis in the S-phase. The passage through the restriction point is primarily controlled by the retinoblastoma protein (pRB)[1] family, which consists of pRB, p107, and p130 (1-4). The pRB-family proteins, acting as negative growth regulators, share a structure termed the "pocket domain" and, through the domain, interact with multiple cellular proteins, most notably the E2F family of transcriptional factors (4-6). E2Fs transactivate a set of genes whose products are critical in S-phase entry and progression (5-8). Upon complex formation, pRB-family proteins neutralize the transcriptional activities of E2Fs (4, 6, 9, 10). Furthermore, the pRB-family-E2F complexes appear to also act as E2F site-specific transcriptional repressors (6, 11-13). Hence, by actively repressing E2F-dependent gene expression, the pRB-family proteins prevent cell-cycle progression from G1 to S.

The pocket function of the pRB-family proteins is considered to be negatively regulated by phosphorylation (14-22). Upon mitogenic stimulation, G1 cyclin-cyclin dependent kinase (CDK) complexes are activated and collaboratively phosphorylate the pRB-family proteins (19-29). Through extensive phosphorylation, the pRB-family proteins lose their ability to form complexes with E2Fs, and the released E2Fs initiate S-phase entry and subsequent progression (4, 8, 14, 30). Accordingly, inactivation of

the pRB-family proteins appears to be an essential prerequisite for traversing the restriction point.

Ectopic expression of pRB-family proteins in a variety of cell types gives rise to G1 cell-cycle arrest (31-35), supporting their crucial roles in preventing S-phase entry. Consistently, pRB and p130 are abundantly expressed in G0/G1-arrested cells in their active, hypophosphorylated forms (4, 19, 20, 36). In contrast, the p107 protein levels are modulated in an opposing manner. In quiescent cells, p107 protein levels are very low, which is at least in part due to E2F-dependent transcriptional repression of the *p107* gene, most probably through the pRB-E2F and/or p130-E2F repressor complex (4, 37). As cells progress toward mid-to-late G1, the levels of p107 increase, and in S-phase cells p107 becomes a predominant pocket protein, although the majority are hyperphosphorylated and hence inactivated (4, 19, 37). These results indicate that low levels of p107 present during mid-to late G1 may play an important role in traversing the restriction point in conjunction with other pocket proteins. Alternatively, p107 may play a unique role among the pocket proteins in cells that have already passed the G1 restriction point.

We have previously shown that in certain hematopoietic cells, including BaF3 and 32D cells, ectopically expressed p130 inhibits the cell-cycle in G1, whereas pRB, even in its phosphorylation-resistant form, fails to do so (34, 38). Given this observation, we wished to know the role of p107,

which is structurally closer to p130 than pRB (4), in hematopoietic cell-cycle regulation. We therefore generated BaF3 lymphoid cells in which wild-type or a non-phosphorylatable and thus constitutively active p107 was inducibly expressed. Here we demonstrate that p107 is capable of inhibiting the S-phase cell-cycle progression. We further provide evidence that p107 is involved in inhibiting the cell-cycle in S-phase cells with DNA damage.

MATERIALS AND METHODS

Construction of Plasmids - A p107 mutant, p107ΔS/T-P, which lacks all of the potential phosphorylation sites by cyclin-CDK was generated from human p107 cDNA by multiple rounds of oligonucleotide-mediated mutagenesis with use of Chameleon site-directed mutagenesis system (Stratagene) according to the manufacturer's instructions (22). In this mutant, threonines-332, -340, -369, -385, -915, -997, serines-368, -515, -615, -640, -650, -749, -762, -964, -975, -988, -1009, and -1041 were substituted with alanine residues. Wild-type and the phosphorylation-resistant p107 were tagged with influenza hemagglutinin (HA) epitope at the carboxy terminus (p107HA and p107ΔS/T-P-HA). cDNAs encoding p107HA and p107ΔS/T-P-HA were inserted into a mammalian expression vector, pSP65SRα2 (39). pOPTET-BSD is an inducible cDNA expression vector having the TcIP promoter and the blasticidin-resistance gene as a drug-selection marker (34, 40). A cDNA encoding p107HA, p107ΔS/T-P-HA or adenovirus E1A 12S (E1A) was subcloned into the pOPTET-BSD vector.

Cell Culture - COS-7 and SAOS-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The 6-1 cell is a BaF3-derived mouse pro-B cell line that stably co-express tTA and LacI (34, 40, 41). Cells were cultured in RPMI1640 medium containing 10% FCS and 20% WEHI-3B-conditioned medium (20% WEHI) as a source of interleukin 3 (IL-3). Stable transfectants

that conditionally express p107HA, p107ΔS/T-P-HA or E1A were created by transfecting pOPTET-BSD-p107HA, pOPTET-BSD-p107ΔS/T-P-HA or pOPTET-BSD-E1A into 6-1 cells by electroporation as described (34, 38). Expression of the cDNA-directed proteins in these transfectants was repressed in medium containing 1 μg/ml tetracycline (Tc) and was induced in medium containing 5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 24 h in the absence of Tc.

Immunoprecipitation and Immunoblot Analysis - COS-7 cells in a 100-mm plate were transfected with 10 μg of each expression plasmid using the DEAE-dextran method. SAOS-2 cells in a 100-mm plate were transfected with 20 μg of each expression plasmid using the calcium-phosphate precipitation method. The transfected cells were harvested 2 days after transfection, and lysed in ELB buffer (250 mM NaCl, 5 mM EDTA, 50 mM HEPES, pH 7.0, 0.5% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsine inhibitor, 0.5 μM DTT and 1 mM phenylmethylsulfonyl fluoride (PMSF) (32). Cell lysates were then immunoprecipitated with anti-HA mouse monoclonal antibody (12CA5) or anti-adenovirus E1A mouse monoclonal antibody (PharMingen, PM-14161A). Immunoprecipitates were recovered on protein A-sepharose beads, washed four times with ELB buffer and eluted by boiling in SDS-containing sample buffer. Immunoprecipitates and lysates were resolved by electrophoresis on a 7.5, 10 or 12% SDS-polyacrylamide gel. Gels were transferred to polyvinyldenedifluoride

membrane filters (Millipore) as described (42) and subjected to immunoblot analysis using anti-HA, anti-p107 (Santa Cruz Biotechnology, sc-318), anti-p130 (sc-317), anti-p53 (sc-1314), anti-$p21^{WAF1}$ (Oncogene Research Products, OP79), anti-cyclin A (sc-751), anti-E2F4 (sc-512) or anti-adenovirus E1A, followed by anti-mouse IgG (Amersham), anti-rabbit IgG (Amersham) or anti-goat IgG (sc-2020) secondary antibody conjugated to horseradish peroxidase. Proteins were visualized using the enhanced chemiluminescence (ECL) detection system (NEN).

Cell-Cycle Analysis - 6-1 cells and their stable transfectants were washed three times with phosphate-buffered saline (PBS) and resuspended at a density of 4×10^5/ml with RPMI1640 medium containing 10% FCS for cytokine starvation. Cells were then divided into two and cultured with IL-3-depleted medium in the presence of 1 μg/ml Tc or 5 mM IPTG. Following 24 h culture in the absence of cytokine, cells were restimulated with IL-3 at a final concentration of 200 pg/ml. At 8 h after IL-3 restimulation, cisplatin was added to the culture at a final concentration of 8 μg/ml. Cells were harvested at appropriate time points.

For cell-cycle synchronization at early S-phase, the 6-1-derived transfectant cells were first starved for cytokine and restimulated with IL-3 in the presence of 1 μg/ml Tc as above described. After 4 h of IL-3 restimulation, they were washed twice with PBS, divided into two and cultured with IL-3-containing medium in the presence of 1 μg/ml Tc or 5

mM IPTG. These cells were respectively treated with hydroxyurea for additional 16 h at a final concentration of 5mM. The cells were then washed twice with PBS to remove HU, and cultured with IL-3-containing medium in the presence of 200 ng/ml nocodazole and 1 μg/ml Tc or 5 mM IPTG.

For flow cytometric analysis, harvested cells were washed in PBS, fixed in 80% ethanol on ice. The cells were washed again and resuspended in PBS containing 500 μg/ml RNase A for 20 min at 37 °C. The samples were incubated for another 15 min at 4 °C with propidium iodide solution (100 μg/ml propidium iodide, 0.1% sodium citrate) prior to flow cytometric analysis with a Becton Dickinson FACS Calibur. Cell-cycle profiles were determined by use of the CELL Quest and ModFit cell-cycle analysis softwares.

RESULTS

Effect of the Phosphorylation-Resistant p107 on the Hematopoietic Cell-cycle 6-1 cell is a BaF3-derived mouse lymphoid cell line whose growth is totally dependent on interleukin 3 (IL-3), with the depletion of IL-3 24 h from the start of culture inducing G0/G1 cell-cycle arrest (Fig. 1A, 0 h). In these growth-arrested 6-1 cells, a member of pRB family, p130, was abundantly expressed whereas expression of p107 was very low (Fig. 1B, lane 1). We have previously shown that the IL-3-dependent growth of the 6-1 cell is inhibited by ectopic expression of p130 but not by pRB, indicating that the cell is actively halted in G0/G1 by the accumulated p130 in the absence of IL-3 and inactivation of p130 is an essential prerequisite to traversing the G1 restriction point in this cell (34).

Restimulation of the growth-arrested 6-1 cells with IL-3 gave rise to synchronized cell-cycle reentry and progression from G1- to S-phases (Fig. 1A). At 8 h after IL-3 stimulation, most of the p130 became hyperphosphorylated (Fig. 1B, lane 2), indicating that by this time G1 cyclin-CDKs were activated sufficiently to phosphorylate and inactivate the pRB-family pocket proteins (4, 19, 20, 22, 36, 37). This in turn suggests that most if not all cells passed through the G1 restriction point by 8 h after the IL-3 stimulation. After 16 h, approximately 80% of cells entered S-phase (Fig. 1A). By this time point, p130 became barely detectable, whereas p107 was potently induced and abundantly expressed but was hyperphosphorylated (Fig. 1B, lane 3). Twenty-four h after IL-3 stimulation, the cell-cycle profile returned to the pattern that is typical of asynchronously growing cells (Fig. 1A).

Using this cell-cycle re-entry/progression protocol, we wished to address the possible role of p107 in cell-cycle regulation, particularly in S-phase progression. As an initial approach, we generated 6-1-deribed stable transfectants, p107-13 and p107-16, that conditionally express wild-type p107 (Fig. 2A) with the use of the tetracycline/IPTG dual-regulated inducible system, in which expression of the cDNA is strongly inhibited by tetracycline and is potently activated by IPTG (34, 40). However, despite ectopic p107 expression (Fig. 2B), we were not able to see any changes in the cell-cycle entry and progression in response to elevated p107 (Fig. 2C). This may be at least in part due to insufficient expression of ectopic p107, as actively cycling 6-1 cells already express high levels of endogenous p107 (Fig. 1B). Indeed, we could only increase the p107 levels by at most two fold in relation to endogenous levels (Fig. 2B, compare the expression of total p107 between Tc- and IPTG-treated lanes at the corresponding times). Furthermore, the massive phosphorylation of p107 in response to IL-3 restimulation (Fig. 2B) indicates that under such circumstances cells possess p107 kinase activity that is sufficient to inactivate both endogenous and exogenous p107.

Accordingly, we next generated a phosphorylation-resistant p107 mutant by replacing all of the serine and threonine residues that constitute possible cyclin-CDK phosphorylation motifs (either serine-proline or threonine-proline) with non-phosphorylatable alanine residues (Fig. 3A) (22). Upon its transient expression in COS-7 cells, the phosphorylation-resistant p107, p107ΔS/T-P-HA, was detected exclusively in its fast-migrating, hypophosphorylated form (Fig. 3B). Furthermore, it formed

physical complexes with endogenous E2F4 and cyclin A in both of COS-7 cells and SAOS-2 osteosarcoma cells (Fig. 3C), indicating that the mutant is biologically active despite multiple point mutations (22).

The cDNA encoding p107ΔS/T-P-HA was introduced into the 6-1 cells and was inducibly expressed in the stable transfectants, PRp107-14 and PRp107-24, with the use of the tetracycline/IPTG dual regulation system (Fig. 4A). The transfectant clones were first growth-arrested in G0/G1 by IL-3 deprivation for 24 h. During IL-3 starvation, cells were also treated with Tc or IPTG. Upon IPTG treatment, the phosphorylation-resistant p107ΔS/T-P-HA was expressed in the growth-arrested cells (Fig. 4B, lane 3), whereas it was undetectable in the same transfectant that was treated with Tc (Fig. 4B, lane 1). Restimulation of the G0/G1-arrest cells with IL-3 in the presence of Tc (i.e. the uninduced condition) gave rise to a cell-cycle progression that is basically indistinguishable from that observed with the parental 6-1 cells (Fig. 4C, Tc; see also Fig. 1A). In striking contrast, the same cells inducibly expressing p107ΔS/T-P-HA exhibited a severe delay of S-phase entry and progression, despite the continued presence of IL-3 for 48 h (Fig. 4, C and D).

To investigate the S-phase inhibitory activity of the phosphorylation-resistant p107 more directly, we examined the effect of p107ΔS/T-P-HA in cells synchronized in the early S-phase (Fig. 5A). G0/G1-arrested PRp107-24 cells were restimulated with IL-3 and, at 4 h after the IL-3 restimulation, the DNA synthesis inhibitor hydroxyurea (HU) was added to the culture at a final concentration of 5mM. At this time, IPTG was also added to the culture to induce p107ΔS/T-P-HA. Since these cells pass through the G1 restriction

point by 8 h after the onset of IL-3 restimulation and 4 h IPTG induction is too short to induce p107ΔS/T-P-HA protein in cells[2], the HU-treated cells were expected to traverse from G1 to S without receiving the effect of the phosphorylation-resistant p107. The cells were treated with HU and IPTG for 16 h to induce sufficient amounts of p107ΔS/T-P-HA while arrested in the early S-phase. As expected, in these HU-blocked cells, endogenous p107 molecules were totally hyperphosphorylated (Fig. 5B, lanes 2, 4). Furthermore, the cells expressed p107ΔS/T-P-HA whose levels were not increased by further incubation with IPTG (Fig. 5B, lanes 4, 5). The early S-phase-synchronized cells were then released from the HU-block and subsequent cell-cycle progression was monitored by flow cytometry in the presence of 200 ng/ml of the tubulin inhibitor nocodazole to prevent entry into the next cell-cycle. In the control cells in which expression of p107ΔS/T-P-HA was not induced, DNA synthesis was rapidly initiated upon HU-release, and completion of full genome replication occurred within 18 h after the release (Fig.5C). In striking contrast, in the presence of p107ΔS/T-P-HA, DNA synthesis was severely impaired and most cells failed to achieve replication of full genome as late as 24 h (Fig. 5C). These results clearly demonstrate that the phosphorylation-resistant p107 is capable of inhibiting progression of the S-phase. It should be noted, however, that the cells were obviously capable of initiating some degree of DNA synthesis in the presence of p107ΔS/T-P-HA although they failed to complete genome replication (Fig. 5C). This may indicate that chain elongation can still take place in the presence of the phosphorylation-resistant p107.

Although the phosphorylation-resistant p107 is capable of inhibiting S-phase progression, it remains possible that the S-phase inhibitory effect is due to supraphysiologic levels of the protein expressed. To address this possibility, we examined the expression levels of the p107 mutant. Because p107ΔS/T-P-HA is detectable exclusively in its hypophosphorylated form, it is possible to compare expression levels between endogenous p107 and p107ΔS/T-P-HA. In the IPTG-treated PRp107-24 cells wherein p107ΔS/T-P-HA was inducibly expressed (Fig. 4B, lanes 3-6, and 5B, lanes 4, 5) and cell-cycle progression was inhibited (Fig. 4, C and D, and 5C), the increase in the levels of the hypophosphorylated form of p107 was marginal and the overall p107 levels elevated only slightly (Fig. 4B and 5B, compare the expression of total p107 between Tc- and IPTG-treated lanes at the corresponding times) as measured by immunoblotting with the use of anti-p107 that recognizes C-terminal 18 amino acids that are perfectly identical between human and mouse p107. These results indicate that there is significantly less expression of the phosphorylation-resistant p107 than that of endogenous p107.

Inhibition of S-phase Progression by Genotoxic Stress - To pursue the biological relevance of the p107-mediated S-phase inhibition, we next addressed whether the hypophosphorylated p107 could be detected in cells that have passed the G1 restriction point. To do so, we arrested 6-1 cells in G0/G1 by IL-3 starvation. The growth-arrested cells were then stimulated with IL-3, and at 8 h after IL-3 stimulation, by which time a majority of cells have passed through the restriction point as judged by the phosphorylation status of pRB-family proteins (see Fig. 1B, lane 2), a DNA damaging agent

cisplatin was added to the culture. Cell-cycle analysis revealed that the cisplatin treatment provoked a delay in S-phase entry that was followed by a severe inhibition of S-phase cell-cycle progression (Fig. 6A), a change that is reminiscent of that observed with the cells expressing the phosphorylation-resistant p107 (see Fig. 4C and 5C). These results indicate that genotoxic stress such as DNA damage can inhibit S-phase cell-cycle progression. We also note that p53 and p21 proteins were upregulated within several hours after cisplatin treatment as reported previously (Fig. 6B) (39).

We also examined the phosphorylation status of p107 in cells treated with cisplatin. Whereas p107 was mostly hyperphosphorylated when cisplatin was added to the culture (8 h after IL-3 stimulation), the hypophosphorylated form of p107 reappeared at 16 h after IL-3 stimulation in the cisplatin-treated cells, but not in untreated cells, and increased thereafter (Fig. 6B, lanes 3, 6-8). This observation suggests that cell-cycle inhibition in S-phase cells is concomitantly associated with the reappearance and accumulation of hypophosphorylated p107.

The Effect of Ectopic p107 on Cellular Responsiveness to Cisplatin - To address the role of p107 in S-phase cell-cycle inhibition in DNA damaged cells more directly, we employed the p107-16 cells that conditionally express HA-tagged, wild-type p107 under the control of the tetracycline/IPTG dual-regulation system (see Fig. 2A). The p107-16 cells were growth-arrested by IL-3 deprivation and then restimulated with IL-3. During IL-3 starvation, p107 was induced by IPTG (Fig. 2B). As previously demonstrated in Fig. 2C, a simple induction of ectopic p107 did not significantly alter the cell-cycle profile of the 6-1 cells. However,

following treatment with cisplatin at 8 h after IL-3 stimulation, p107-induced cells exhibited a stronger inhibition of S-phase progression than that observed in p107-uninduced cells (Fig. 7A). These results indicate that the cell-cycle inhibitory activity of cisplatin is proportional to the p107 levels. Intriguingly, in these cisplatin-treated cells, ectopic p107 existed most exclusively in its hypophosphorylated form as late as 24 h after IL-3 stimulation (Fig. 7B, lane 10). Because the HA-tagged p107 is constitutively produced under the TcIP promoter in the transfectants, our results indicate that phosphorylation of p107 may be inhibited in the DNA-damaged S-phase cells.

Effect of E1A Oncoprotein on the S-phase Inhibitory Effects of Cisplatin - Adenovirus E1A protein is known to physically interact with and functionally inactivate pRB-family pocket proteins (43-46), including p107 (33, 47, 48). Accordingly, if p107 is actively involved in the inhibition of S-phase cell-cycle progression in response to DNA damage, then E1A would be expected to counteract the cell-cycle inhibitory activity of cisplatin. To address this, we generated a 6-1-derived, stable transfectant, E1A15-1, that ectopically expresses the E1A 12S product (Fig. 8A). Expectedly, in the E1A transfectant cell, the E1A protein formed physical complexes with endogenous p107 (Fig. 8B). However, a simple overexpression of E1A did not provoke any significant change in IL-3-dependent growth in 6-1 cells[3]. The parental and the E1A-expressing cells were then growth-arrested by IL-3 deprivation and were subjected to cisplatin treatment at 8 h after IL-3 restimulation. In cells expressing E1A, the S-phase inhibitory activity of cisplatin was significantly reduced and a relatively large fraction of cells

was capable of exiting from S-phase within 32 h (Fig. 8, C and D). These results provide additional evidence that p107 is actively involved in the cell-cycle inhibition induced by DNA damage in S-phase cells.

DISCUSSIONS

In mammalian cells, the point of decision regarding the move towards quiescence or proliferation occurs at the G1 restriction point (49). A series of works have highlighted the critical role of pRB-family proteins in traversing the G1 restriction point (1, 2), raising the possibility that pRB-family proteins may be dispensable in cell-cycle regulation beyond the G1 restriction point. Consistent with this idea, once cells pass through the restriction point, they are systemically inactivated by phosphorylation and are kept inactive throughout subsequent cell-cycle phases. When cells enter the next G1-phase, they are converted into their hypophosphorylated form by phosphatases (50, 51). This scenario may well be applicable to cells that continuously proliferate. However, factors such as DNA damage can activate otherwise concealed cell-cycle checkpoints and arrest cells in post-G1 (52). It is therefore possible that, under certain cellular settings, the pRB-family pocket proteins may play an active role in post G1 cell-cycle regulation.

The expression levels of pRB are relatively constant throughout the cell-cycle (4, 19). In contrast, the levels of 107 and p130 proteins change dramatically as cells progress through the cell-cycle. p130 is abundantly present in quiescent cells and, upon mitogenic stimulation, it is hyperphosphorylated in mid to late G1-phase. The p130 protein levels are then progressively downregulated as cells move into S-phase (4, 20, 36). In contrast, the levels of p107 are regulated in a manner opposite to that observed for p130. In quiescent cells, p107 expression is very low. As the cell-cycle progresses from the early to the mid/late G1-phases, p107 begins to accumulate dramatically (4, 19). This p107 expression is primarily

regulated at the transcriptional level (8). In particular, reciprocal expression between p107 and p130 can be explained at least in part by the E2F sites present in the promoter of the *p107* gene (37). It has been suggested that the *p107* promoter is under the negative control of p130-E2F and/or pRB-E2F complex present in the quiescent cells. Increases in p107 levels as the cell-cycle progresses from the quiescent state again suggests that the pocket protein might be able to regulate post-G1 cells, rather than simply preventing S-phase entry.

We have previously shown that growth of hematopoietic cells such as BaF3 and 32D is strongly inhibited by ectopic expression of p130, but not by pRB, even in its phosphorylation-resistant form (34, 38). In these cells, p130 prevents S-phase entry, at least in part, through inhibiting the transcriptional activity of E2F-4 (34). Because p107 is more similar to p130 than pRB among the pRB-family pocket proteins (4, 53), we wished to determine the role of p107 in the hematopoietic cell-cycle. As an initial approach, we generated 6-1 transfectants that conditionally express wild-type p107 through the use of the Tc/IPTG dual-regulated, inducible promoter system (34, 40). Because p107 is a relatively abundant protein in cycling cells, we could only increase the cellular p107 levels at most by two fold upon induction. Under this condition, we were not able to observe any changes in IL-3-dependent cell-cycle entry and progression by the elevated p107. Possibly, the amounts of ectopic p107 expressed were not sufficient to exert biological activities. Alternatively, p107 might be much more sensitive to G1 cyclin-CDK than pRB and p130, and may be instantly inactivated during

G1 progression. If the latter is correct, then we can conclude that p107 does not play a major role in G1 cell-cycle regulation.

To pursue further the possible role of p107 in cell-growth regulation, we generated in this work a novel p107 molecule that is completely resistant to cyclin-CDK mediated phosphorylation/inactivation. Upon its inducible expression in the BaF3-derived cells, we observed two distinct effects of the phosphorylation-resistant p107 in cell-cycle regulation. First, the phosphorylation-resistant p107 is capable of retarding the G1-to-S transition. Because p107 preferentially binds E2F-4 and E2F-5 (5, 54, 55), it is reasonable that the phosphorylation-resistant p107 would inhibit G1 cell-cycle progression when ectopically expressed in G1 cells, as is the case with p130 (33, 37). Second, the p107 mutant was found to strongly inhibit S-phase progression. Indeed, in the presence of the phosphorylation-resistant p107, virtually no cells exited from S-phase by as late as 48 h after IL-3 stimulation. Because the levels of phosphorylation-resistant p107 required to induce S-phase inhibition were significantly less than those of endogenous p107, we conclude that the p107-mediated S-phase inhibition indeed takes place when a certain fraction of the endogenous p107 is converted from the hyperphosphorylated form to the hypophosphorylated form. The observation that only the hypophosphorylated p107 is capable of inhibiting S-phase progression indicates that the activity is the pocket structure, but not the spacer domain, dependent.

The biological relevance of the S-phase inhibitory activity of p107 was provided by the observation that the DNA-damaging agent cisplatin is capable of inhibiting an S-phase progression that is concomitantly associated

with the accumulation of the hypophosphorylated form of endogenous p107. This finding suggests that conditions exist that result in the appearance of active p107 beyond the restriction point in late G1. Furthermore, cisplatin was found to inhibit S-phase cell-cycle progression more effectively as cells expressed higher levels of p107. Conversely, the inhibition of p107 activity by the overexpression of the adenovirus E1A 12S product, which binds and inactivates pRB-family proteins (33, 43-48), at least partially restored the S-phase inhibitory activity of cisplatin. Together with p107 being a predominantly expressed pRB-family protein in S-phase cells (4, 19), our results indicate that p107 is involved in the inhibition of S-phase progression in response to DNA damage.

Although the mechanism through which hypophosphorylated p107 accumulates in cisplatin-treated cells needs further investigation, our observation implies that a p53-p21 pathway (56-58) may be involved. Upon cisplatin treatment, p53 and p21 proteins are upregulated in the 6-1 cells as has been previously demonstrated (39). Because p21 acts as a universal inhibitor of cyclin-CDK (59, 60), p107 phosphorylation may be inhibited in the presence of p21 in the cisplatin-treated cells. Consequently, *de novo*-synthesized p107 would remain hypophosphorylated because of the reduced p107 kinase activity. This possibility is supported by our finding that in cisplatin-treated cells, the newly synthesized p107 molecules, which can be marked by an HA epitope tag, are detectable almost exclusively in their hypophosphorylated form. It is also possible that p107 phosphatases are induced in the cisplatin-treated cells, converting hyperphosphorylated p107 to less phosphorylated forms. In this regard, p107 was shown to be rapidly

dephosphorylated by protein phosphatase 2A (PP2A) upon UV irradiation (61).

Recent studies have shown that overexpression of phosphorylation-resistant pRB mutants in fibroblasts is capable of inhibiting S-phase progression (62-64). This S-phase inhibitory activity is not overcome by E2F or cyclin A/E (62, 63), indicating that pRB is capable of regulating the S-phase cell-cycle through the mechanisms independent of E2F. Taken together, the two pRB-family pocket proteins, pRB and p107, may function in a combinatorial way as S-phase cell-cycle brakes that are turned on in response to DNA damage in cells that have passed the G1 restriction point. Probably, the relative contributions of these two proteins in S-phase response may vary among cell types, as pRB even in its constitutively active form is incapable of effectively inhibiting cell-cycle progression in BaF3 cells (34).

The molecular mechanisms through which p107 or pRB inhibits S-phase progression is currently unknown. In the case of pRB, the S-phase response is suggested to involve origin-activation machineries (65). As is the case of pRB, the early-S-phase-synchronized cells were capable of initiating some degree of DNA synthesis but were incapable of full genome replication in the presence of phosphorylation-resistant p107. This finding is consistent with the idea that p107 inhibits the origin activation process rather than the DNA elongation process (66). In this regard, it has been recently reported that pRB family proteins (pRB, p107, and p130) can bind MCM7, a component of pre-replication complex (pre-RC), and inhibit *in vitro* DNA replication in an MCM7-dependent manner (65). It is therefore

intriguing to speculate that hypophosphorylated p107 is capable of inhibiting origin activation by directly interacting with MCM or the related pre-RC components. Indeed, pRB family members are associated with early S-phase replication sites (67). Alternatively, p107 may inhibit S-phase progression through transcriptional control because ORC1 and CDC6 genes, whose products are also essential components of pre-RC, are transcriptionally activated by E2F, the critical target of the pRB family proteins (68, 69).

The involvement of p107 in S-phase cell-cycle arrest in response to DNA damage indicates that its functional loss may provoke abnormal cell-cycle progression that is concomitantly associated with genetic instability.

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[1]The abbreviations used are : pRB, retinoblastoma protein; CDK, cyclin-dependent kinase; HA, hemagglutinin; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; ELB, E1A lysis buffer; ECL, enhanced chemiluminescence; S/T-P motif, serine-proline or threonine-proline motif; Tc, tetracycline; IPTG, isopropyl β-D-thiogalactopyranoside; HU, hydroxyurea.

[2]T. Kondo, and M. Hatakeyama, unpublished observations.

[3]T. Kondo, and M. Hatakeyama, unpublished observations.

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FIGURE LEGEND

FIG. 1. **Expression of pRB-Family Proteins during Cell-Cycle Entry and Progression of 6-1 Cells.** BaF3-derived 6-1 cells were growth-arrested by IL-3 starvation for 24 h and then restimulated with IL-3. *A*, cell-cycle kinetics of the IL-3-restimulated 6-1 cell. Cells were harvested at the indicated time points after IL-3 restimulation, stained with propidium iodide, and DNA contents were measured by flow cytometry. *B*, expression and phosphorylation of p107 and p130 in the IL-3-stimulated 6-1 cells. Whole cell lysates were prepared at the indicated time points after IL-3 restimulation, resolved on 7.5% SDS-PAGE, and analyzed by immunoblotting with anti-p107 (upper panel) or anti-p130 (lower panel). (pp) indicates a hyperphosphorylated form of p107 or p130, whereas (p) indicates a hypophosphorylated form of p107 or p130.

FIG. 2. **Effect of Ectopic p107 Expression on the Cell-Cycle Entry and Progression of 6-1 Cells.** *A*, induction of the HA-tagged, wild type p107 in the 6-1-derived stable transfectant clones, p107-13 and p107-16. The stable transfectants were cultured in IL-3-containing medium in the presence of tetracycline (Tc; uninduced condition) or IPTG (induced condition) for 24 h. Whole cell lysates were prepared, resolved on 7.5% SDS-PAGE and analyzed by immunoblotting with anti-HA. Asterisk indicates a non-specific

band. *B*, induced expression of ectopic p107 during cell-cycle entry and progression. The p107-16 cells were growth-arrested by IL-3 starvation for 24 h in the presence of Tc or IPTG and restimulated with IL-3. Whole cell lysates were prepared at the indicated time points after IL-3 restimulation, resolved on 7.5% SDS-PAGE, and analyzed by immunoblotting with anti-HA which specifically detects ectopic p107 (upper panel) or anti-p107 which recognizes both endogenous and ectopic p107 molecules with the same affinity (lower panel). (pp) indicates a hyperphosphorylated form, whereas (p) indicates a hypophosphorylated form. Asterisk shows a non-specific band. *C*, cell-cycle entry and progression in the presence or absence of ectopic p107. The p107-16 cells were growth-arrested by IL-3 starvation for 24 h in the presence of Tc or IPTG and then restimulated with IL-3, harvested at the indicated time points after IL-3 restimulation, stained with propidium iodide, and DNA contents were measured by flow cytometry.

FIG. 3. **Biological Properties of the Phosphorylation-Resistant p107 Mutant, p107ΔS/T-P-HA.** *A*, schematic view of p107ΔS/T-P-HA that lacks all of the potential cyclin-CDK phosphorylation sites. A and B boxes are conserved regions among pRB family proteins. The phosphorylation-resistant p107 was tagged with the HA epitope at the C-terminus. *B*, expression of p107ΔS/T-P-HA in COS-7 cells. Whole cell lysates of COS-7 cells transiently transfected with an empty vector, pSP65SRα2 (lane 1), an

expression vector for the HA-tagged, wild type p107, pSP65SRα2-p107HA (lane 2), or a p107ΔS/T-P-HA expression vector, pSP65SRα2-p107ΔS/T-P-HA (lane 3), were analyzed by immunoblotting with anti-HA. (pp) indicates a hyperphosphorylated form, whereas (p) indicates a hypophosphorylated form. *C,* Physical interaction of p107ΔS/T-P-HA with cyclin A and E2F4 *in vivo*. Whole cell lysates were prepared from COS-7 cells (left panel) or SAOS-2 cells (right panel) transiently transfected with pSP65SRα2 (lane 1, 4), pSP65SRα2-p107HA (lane 2, 5) or pSP65SRα2-p107ΔS/T-P-HA (lane 3, 6). The lysates were immunoprecipitated with anti-HA (lanes 4-6) and the immunoprecipitates were analyzed on immunoblotting with anti-cyclin A (upper panel; lanes 4-6) or anti-E2F4 (lower panel; lanes 4-6). Anti-cyclin A and anti-E2F4 immunoblottings of the whole cell lysates are shown on lanes 1-3.

FIG. 4. **Inhibition of S Phase Entry and Progression by the Ectopic Expression of p107ΔS/T-P-HA.** *A,* induction of p107ΔS/T-P-HA in the 6-1-derived stable transfectant clones, PRp107-16 and PRp107-24. Cells were cultured in IL-3-containing medium in the presence of Tc (uninduced condition) or IPTG (induced condition) for 24 h. Whole cell lysates were prepared, resolved on 7.5% SDS-PAGE and analyzed on anti-HA immunoblotting. (p) indicates a hypophosphorylated form of p107. *B,* inducible expression of p107ΔS/T-P-HA during cell-cycle entry and

progression. The PRp107-24 cells were growth-arrested by IL-3 starvation for 24 h in the presence of Tc or IPTG and restimulated with IL-3. Whole cell lysates were prepared at the indicated time points after IL-3 restimulation, resolved on 7.5% SDS-PAGE, and analyzed on immunoblotting with anti-HA which specifically detects p107ΔS/T-P-HA (upper panel) or anti-p107 which recognizes both endogenous and ectopic p107 molecules with the same affinity (lower panel). (p) indicates a hypophosphorylated form. *C,* effect of p107ΔS/T-P-HA on the cell-cycle entry and progression. The PRp107-24 cells were growth-arrested by IL-3 starvation for 24 h in the presence of Tc or IPTG and restimulated with IL-3, harvested at the indicated time points after IL-3 restimulation, stained with propidium iodide, and DNA contents were measured by flow cytometry. DNA histograms shown are representative of three independent experiments. Essentially the same result was obtained with another stable transfectant clone, PRp107-14. *D,* inhibition of S-phase progression by p107ΔS/T-P-HA. Percentage of cells in each cell-cycle phase in the presence or absence of p107ΔS/T-P-HA was calculated from the flow cytometric data shown in (*C*) with the use of ModFit cell-cycle analysis software. Data are representative of three independent experiments.

FIG. 5. Cell-Cycle Effect of the Phosphorylation-Resistant p107 Expressed in Early S-phase Cells. *A,* S-phase cell-cycle synchronization

protocol. The PRp107-24 cells were growth arrested in the early S-phase by hydroxyurea (HU) treatment. The phosphorylation-resistant p107 was inducibly expressed in the S-phase arrested cells by IPTG treatment. The HU-block was then cancelled by eliminating HU from the culture and the subsequent cell-cycle progression was examined in the presence of nocodazole, which prevents next cell cycle entry. *B*, inducible expression of p107ΔS/T-P-HA in cells specifically synchronized in the early S-phase. Whole cell lysates were prepared at the appropriate time points shown in (*A*), resolved on 7.5% SDS-PAGE, and analyzed on immunoblotting with anti-HA which specifically detects p107ΔS/T-P-HA (upper panel) or anti-p107 which recognizes both endogenous and ectopic p107 molecules with the same affinity (lower panel). (pp) indicates a hyperphosphorylated form, whereas (p) indicates a hypophosphorylated form of p107. *C*, effect of p107ΔS/T-P-HA on S-phase cell-cycle progression. The same PRp107-24 cells used in (B) were stained with propidium iodide. DNA contents were measured by flow cytometry. DNA histograms shown are representative of three independent experiments. Essentially the same result was obtained with another stable transfectant clone, PRp107-14.

FIG. 6. **Effect of DNA Damaging Agent, Cisplatin, on S-Phase Cell-Cycle Progression.** The 6-1 cells were growth-arrested by IL-3 starvation for 24 h and then restimulated with IL-3. At 8 h after IL-3 restimulation, cells were

treated with cisplatin at a final concentration of 8 mg/ml. The cisplatin-treated and untreated cells were harvested at the indicated time points after IL-3 stimulation. *A*, effect of cisplatin on the cell-cycle entry and progression. The cells were stained with propidium iodide, and DNA contents were measured by flowcytometry. *B*, Effect of cisplatin on the expression of cell-cycle regulatory molecules. Whole cell lysates prepared at the each time point were resolved on 7.5% (for p107 and p130) or 12% (for p53 and p21) SDS-PAGE and analyzed by immunoblotting with anti-p107, anti-p130, anti-p53 or anti-p21. (pp) indicates a hyperphosphorylated form of p107 or p130, whereas (p) indicates a hypophosphorylated form of p107 or p130.

FIG. 7. **Effect of Ectopic p107 Expression on Cellular Responsiveness to Cisplatin.** A p107HA transfectant clone, p107-16, was growth-arrested by IL-3 starvation for 24 h in the presence of Tc or IPTG and then restimulated with IL-3. Following 8 h IL-3 stimulation, cells were treated with 8 mg/ml cisplatin for 2 h and cultured in the IL-3-containing medium for indicated periods. *A*, cell-cycle profiles of the cisplatin-treated cells in the presence or absence of ectopic p107. The p107-16 cells were harvested at the indicated time points after IL-3 restimulation, stained with propidium iodide, and DNA contents were measured by flow cytometry. *B*, inducible expression of ectopic p107 during cell-cycle entry and progression of the p107-16 cells in

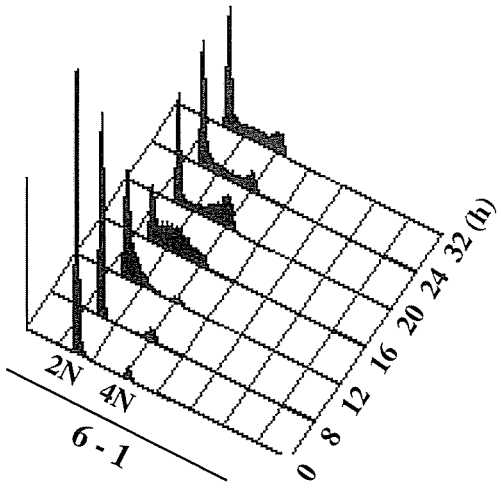
the presence or absence of cisplatin. Whole cell lysates were prepared from the cisplatin-treated and untreated cells at the indicated time points after IL-3 restimulation, resolved on 7.5% (for p107HA) or 12% (for p53 and p21) SDS-PAGE, and analyzed by immunoblotting with anti-HA which specifically detects ectopic p107, anti-p53 or anti-p21. (pp) indicates a hyperphosphorylated form of p107, whereas (p) indicates a hypophosphorylated form of p107.

FIG. 8. **Effect of E1A Oncoprotein on the S-Phase Inhibitory Activity of Cisplatin.** The parental 6-1 and a 6-1 transfectant, E1A15-1, that inducibly expresses adenovirus E1A oncoprotein were growth-arrested by IL-3 deprivation in the presence of IPTG. After 24 h IL-3 starvation, cells were restimulated with IL-3. At 8 h after IL-3 restimulation, they were treated with cisplatin at a final concentration of 8 mg/ml. The cisplatin-treated and untreated cells were harvested at the indicated time points. *A,* whole cell lysates were prepared from the cisplatin-treated and untreated cells, resolved on 7.5% (for p107) or 10% (for p53 and E1A) SDS-PAGE and analyzed by immunoblotting with anti-p107, anti-p53 or anti-E1A. (pp) indicates a hyperphosphorylated form of p107, whereas (p) indicates a hypophosphorylated form of p107. *B,* complex formation between E1A and p107 in cells. whole cell lysates of the parental 6-1 and the E1A-expressing cells harvested at 24 h were subjected to immunoprecipitation with anti-E1A

monoclonal antibody. The E1A-immunoprecipitates were then analyzed on immunoblotting with anti-p107. *C*, cell-cycle profiles of cisplatin-treated cells in the presence or absence of E1A. The parental and the E1A expressing transfectant cells were harvested at the indicated time points after IL-3 restimulation, stained with propidium iodide, and DNA contents were measured by flow cytometry. *D*, S-phase inhibitory activity of cisplatin was reduced in cells expressing E1A. Percentage of cells in each cell-cycle phase was calculated from the flow cytometric data shown in (*C*) with the use of ModFit cell-cycle analysis software. Data are representative of three independent experiments.

Fig.1

A



B

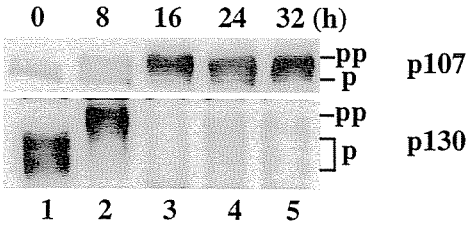
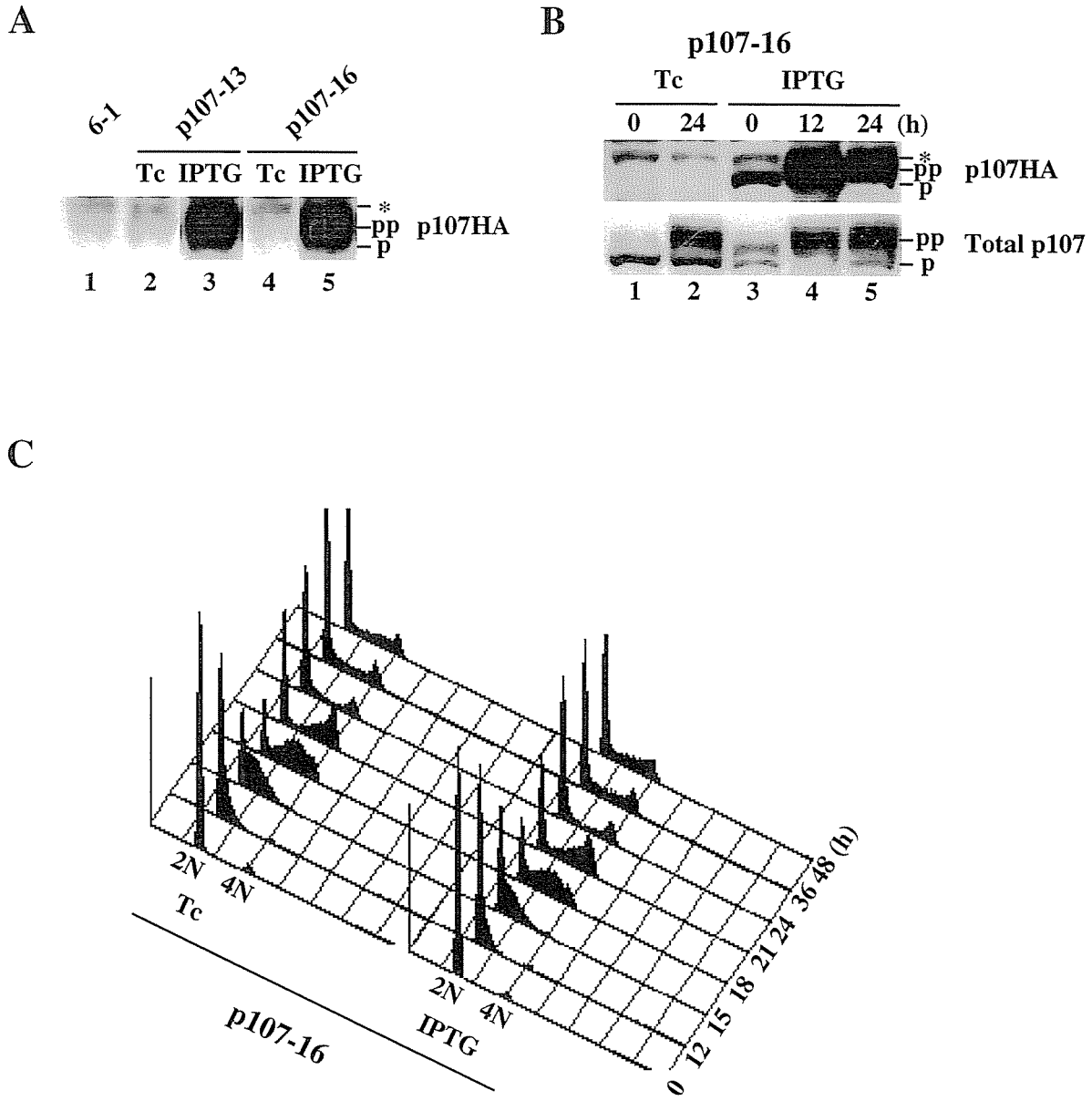


Fig.2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p107-16
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p107HA
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B
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Tc
IPTG
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p
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Tc
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4N
IPTG
p107-16
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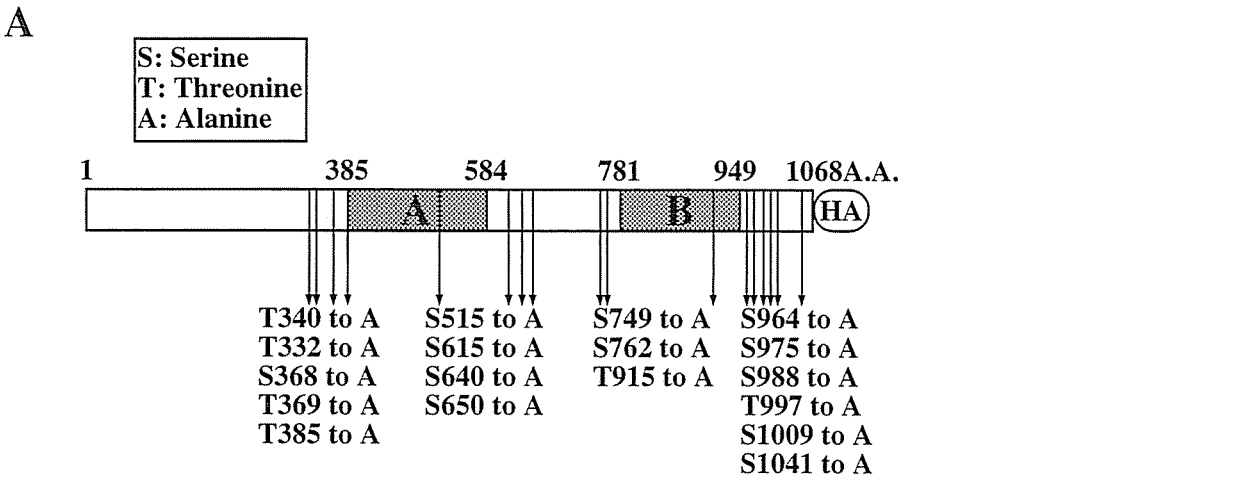
Fig.3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T369 to A
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S515 to A
S615 to A
S640 to A
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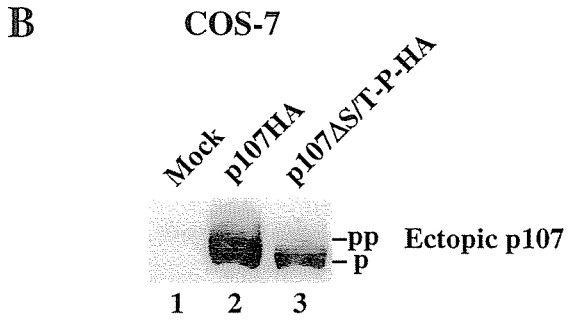
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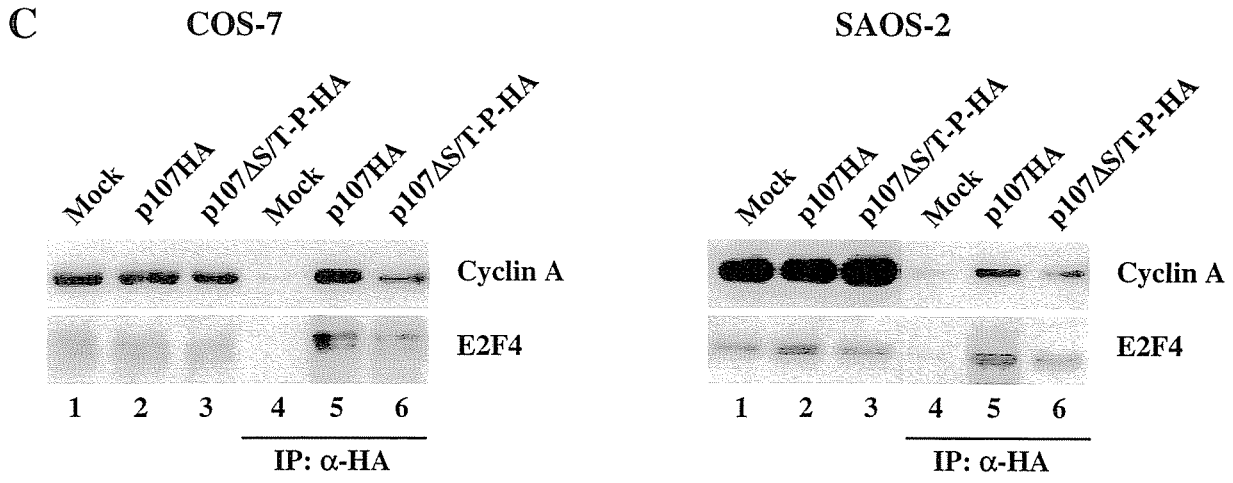
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2
3
4
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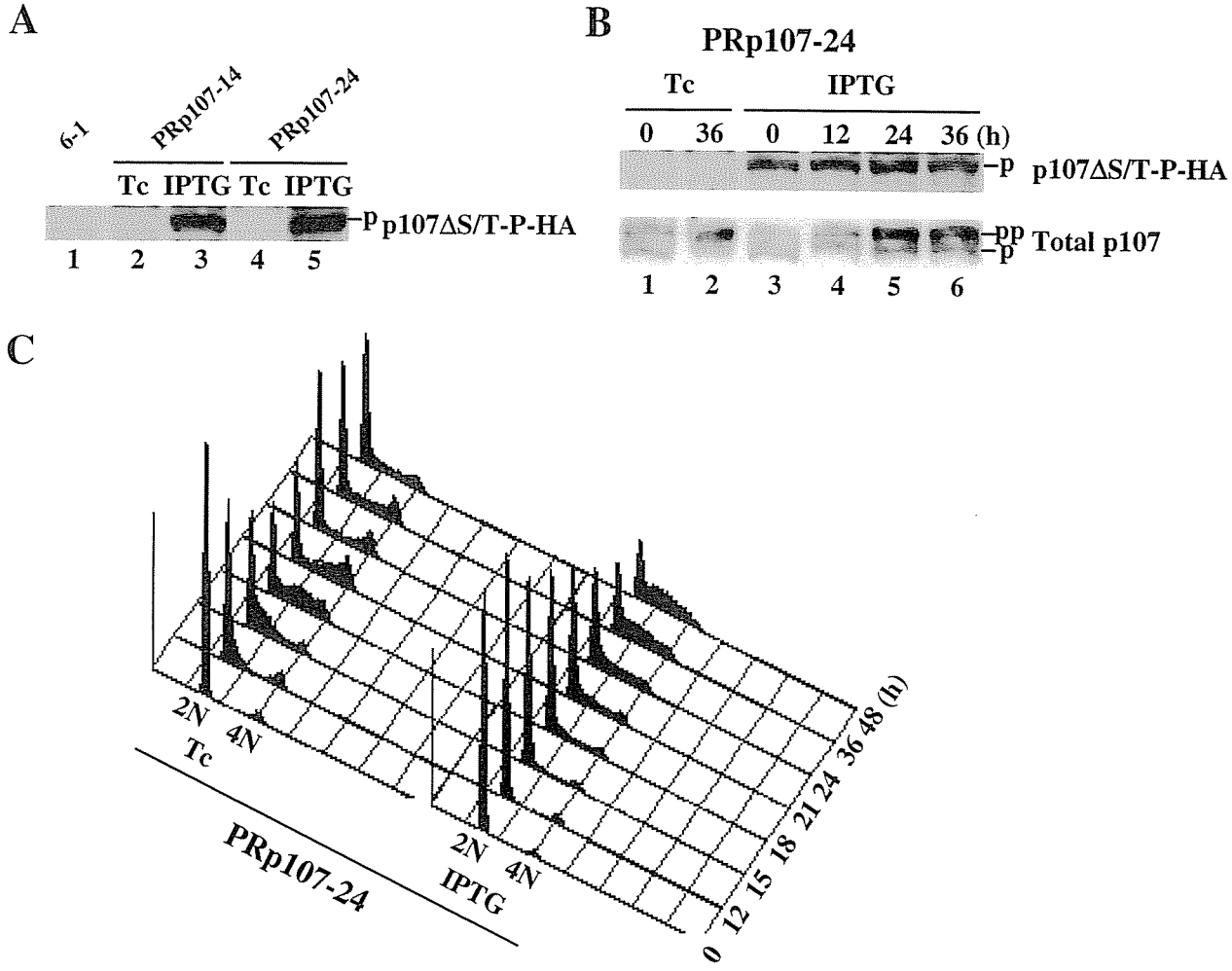
Fig.4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p107ΔS/T-P-HA
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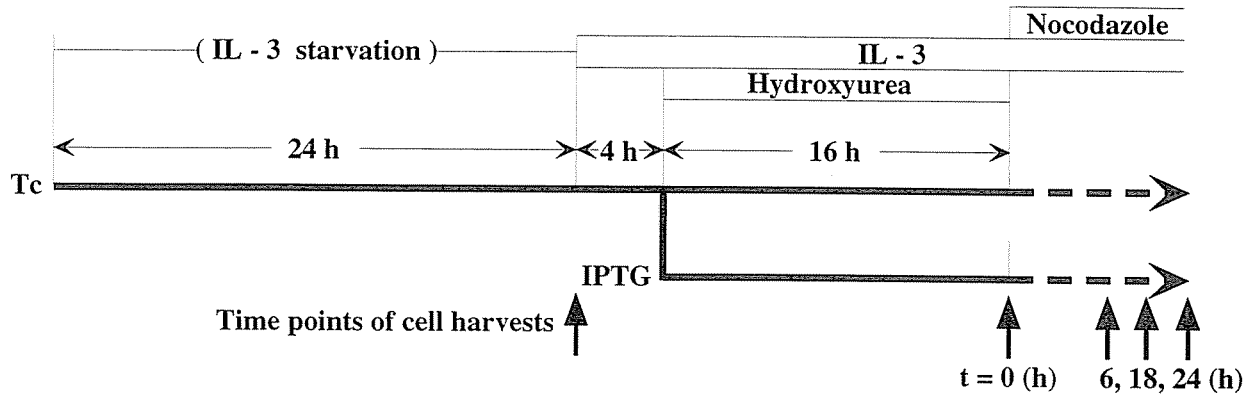
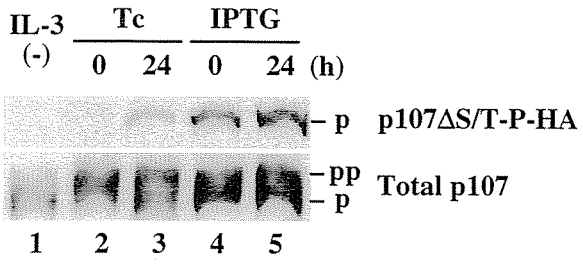
B
PRp107-24
Tc
IPTG
0 36 0 12 24 36 (h)
p107ΔS/T-P-HA
pp
p
Total p107
1 2 3 4 5 6
C
2N 4N
Tc
PRp107-24
IPTG
0 12 15 18 21 24 36 48 (h)
D
(%)
80
70
60
50
40
30
20
10
0
24 h
36 h
48 h
G0/G1
S phase
G2/M
Tc IPTG Tc IPTG Tc IPTG

Fig.5

A


(IL - 3 starvation)
Nocodazole
IL - 3
Hydroxyurea
24 h
4 h
16 h
Tc
IPTG
Time points of cell harvests
t = 0 (h)
6, 18, 24 (h)

B


IL-3
(-)
Tc
IPTG
0 24 0 24 (h)
p
p107ΔS/T-P-HA
pp
p
Total p107
1 2 3 4 5

C

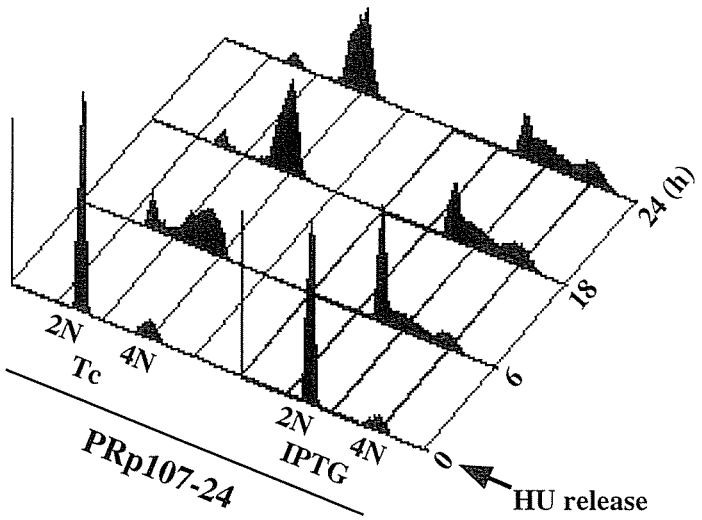
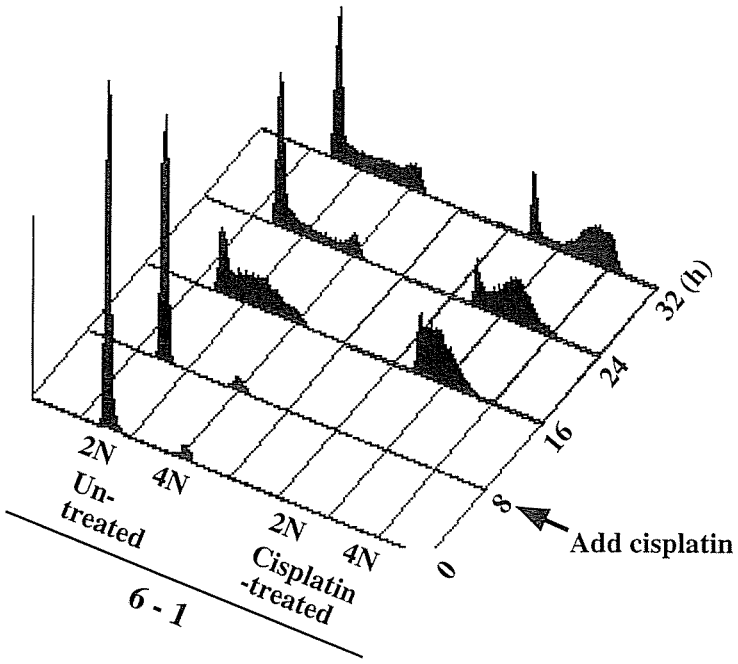

2N
4N
Tc
2N
4N
IPTG
PRp107-24
0
6
18
24 (h)
HU release

Fig.6

A


2N
4N
2N
4N
Un-
treated
Cisplatin
-treated
6 - 1
0
8
16
24
32 (h)
Add cisplatin

B

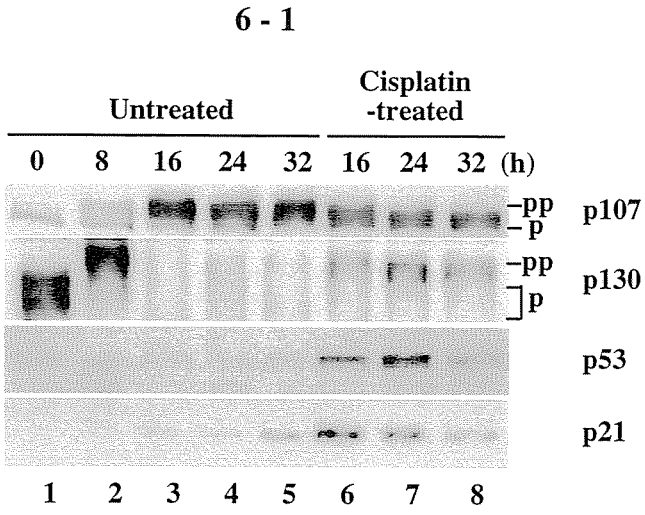
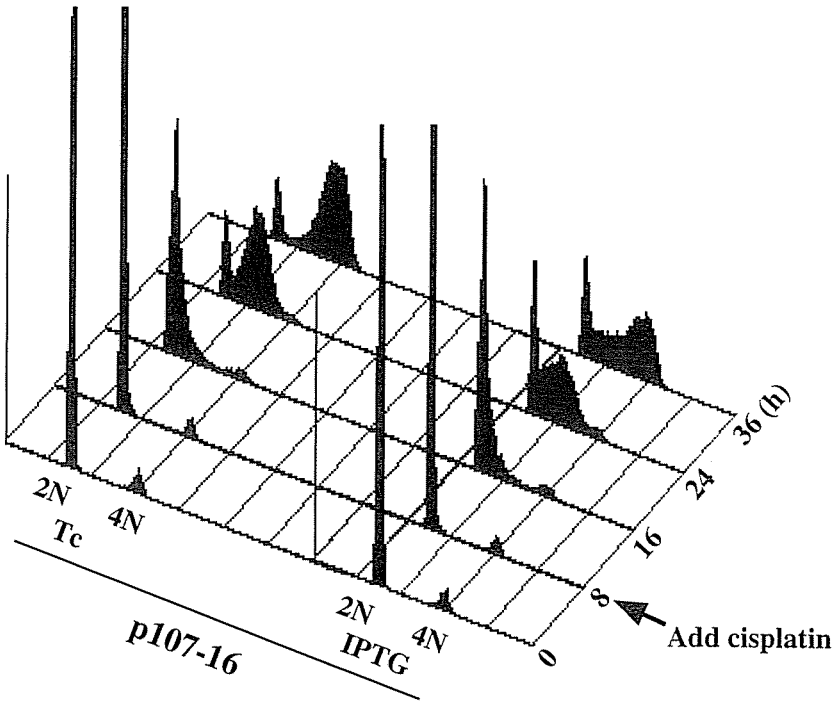

6 - 1
Untreated
Cisplatin
-treated
0 8 16 24 32 16 24 32 (h)
pp
p
p107
pp
p
p130
p53
p21
1 2 3 4 5 6 7 8

Fig.7

A


2N
4N
Tc
p107-16
2N
4N
IPTG
0
8
16
24
36 (h)
Add cisplatin

B

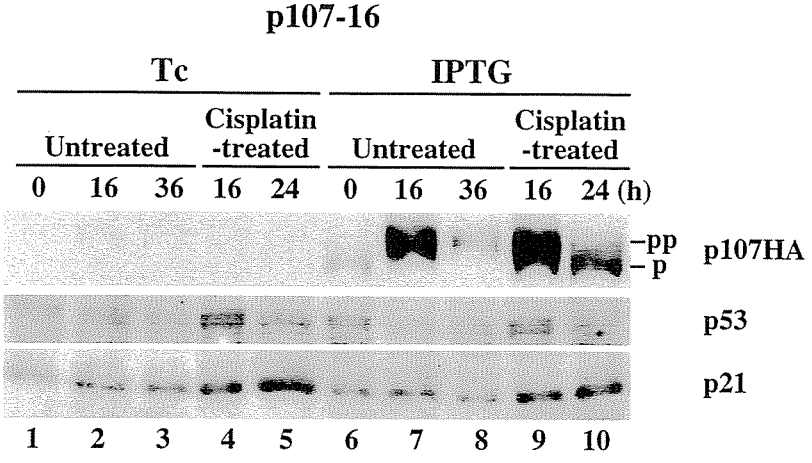
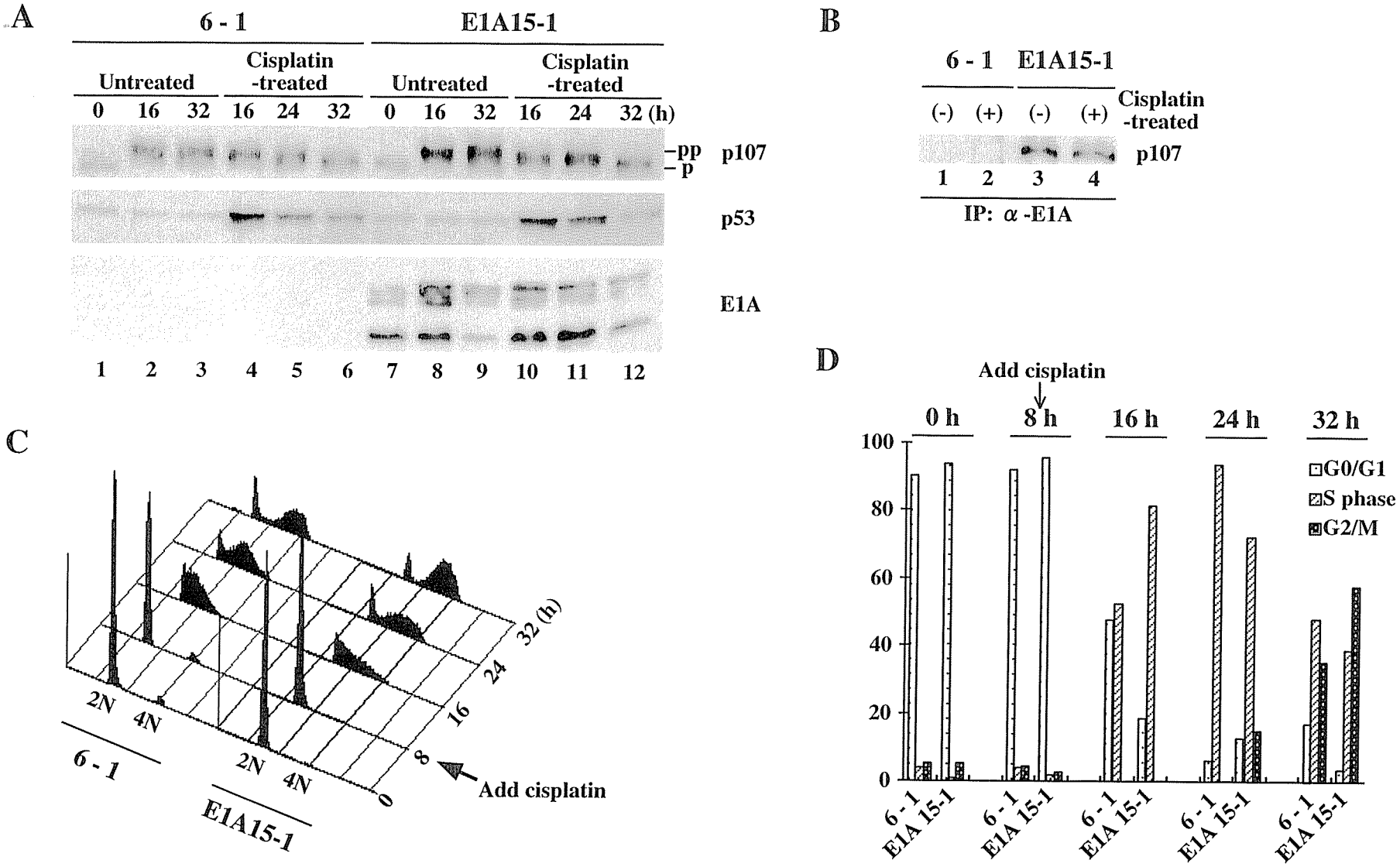

p107-16
Tc
IPTG
Untreated
Cisplatin -treated
Untreated
Cisplatin -treated
0 16 36 16 24 0 16 36 16 24 (h)
pp
p
p107HA
p53
p21
1 2 3 4 5 6 7 8 9 10

Fig.8


A
6 - 1
E1A15-1
Untreated
Cisplatin -treated
Untreated
Cisplatin -treated
0 16 32 16 24 32 0 16 32 16 24 32 (h)
pp
p
p107
p53
E1A
1 2 3 4 5 6 7 8 9 10 11 12
B
6 - 1 E1A15-1
(-) (+) (-) (+) Cisplatin -treated
p107
1 2 3 4
IP: α -E1A
C
2N 4N 2N 4N
6 - 1
E1A15-1
0 8 16 24 32 (h)
Add cisplatin
D
Add cisplatin
0 h 8 h 16 h 24 h 32 h
100 80 60 40 20 0
G0/G1
S phase
G2/M
6 - 1 E1A 15-1