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WAF1 Retards S-Phase Progression Primarily by Inhibition of Cyclin-Dependent Kinases

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The p21$^{WAF1/CIP1/sdi1}$ gene product (WAF1) inhibits DNA replication in vitro (J. Chen, P. Jackson, M. Kirschner, and A. Dutta, Nature 374:386–388, 1995; S. Waga, G. Hannon, D. Beach, and B. Stillman, Nature 369:574–578, 1994), but in vivo studies on the antiproliferative activity of WAF1 have not resolved G_1-phase arrest from potential inhibition of S-phase progression. Here, we demonstrate that elevated WAF1 expression can retard replicative DNA synthesis in vivo. The WAF1-mediated inhibitory effect could be antagonized by cyclin A, cyclin E, or the simian virus 40 small-t antigen with no decrease in the levels of WAF1 protein in transfected cells. Proliferating-cell nuclear antigen (PCNA) overexpression was neither necessary nor sufficient to antagonize WAF1 action. Expression of the N-terminal domain of WAF1, responsible for cyclin-dependent kinase (CDK) interaction, had the same effect as full-length WAF1, while the PCNA binding C terminus exhibited modest activity. We conclude that S-phase progression in mammalian cells is dependent on continuing cyclin and CDK activity and that WAF1 affects S phase primarily through cyclin- and CDK-dependent pathways.

While the mechanisms by which G_1-S and G_2-M transitions are regulated in mammalian cells have been intensively investigated (9, 32), relatively little is known concerning controls that may be operative within S phase. The orderly firing of replication origins, in particular such that each origin is replicated once and only once during S phase, is presumably under strict control (5). Likewise, mechanisms must exist for halting S phase in case of exogenous insults that induce DNA damage. Potential targets for the regulation of S phase include components of the basal replication machinery (e.g., replication protein A, replication factor C, and proliferating-cell nuclear antigen [PCNA]) as well as molecular complexes which putatively license origins of DNA replication. In addition, gene products normally implicated in G_1 or G_2-M control may possess S-phase-regulatory activity under appropriate circumstances. In the latter category, cyclin-dependent kinases (CDKs), in particular, complexes of CDK2 with cyclin A or E, are plausible candidates (8, 14, 24).

Insofar as cyclin-CDK complexes may affect S-phase progression, CDK inhibitors such as p27^{KIP1}, p21$^{WAF1/CIP1/sdi1}$, and p16 must also be considered as candidates for S-phase regulation. These inhibitors have emerged as key players in the regulation of the cell cycle, involved in modulation of G_1-S transit in cases of p53-mediated DNA damage responses, replicative senescence, terminal differentiation, and the antiproliferative action of transforming growth factor β (6, 16, 22, 25, 26). Of particular interest is p21$^{WAF1/CIP1/sdi1}$, since the protein encoded by this plasmid has been reported to affect not only the G_1-S transition but also DNA replication in vitro (3, 10, 37). The in vitro results, while suggestive, do not establish whether WAF1 can in fact affect S-phase progression in vivo. To resolve this issue, we developed a new approach that allows us to study the role of the cyclin and CDK machinery specifically in relation to S-phase control. Our findings reveal that, in intact human cells, WAF1 independently retards the G_1-S transition and S-phase progression. Different domains of WAF1, involved in the inhibition of CDKs and PCNA, have been proposed as having the potential to induce S-phase inhibition. This issue was also examined, and evidence indicating that WAF1 affects S-phase progression primarily through the cyclin- and CDK-dependent pathway was obtained.

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MATERIALS AND METHODS

Cell culture. WI-38 human lung embryo fibroblasts and SAOS-2 cells were obtained from the American Type Culture Collection and propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco BRL).

Expression plasmids. The PCNA expression plasmid, pCMV-PCNA, was constructed by amplification of the PCNA coding region from PCNA-OB (17), followed by insertion into pCMV.3 (23). (A second PCNA plasmid, based on the expression vector pX, obtained from M. Pagano, was tested, and the same results were obtained [data not shown].) Identities of other expression vectors were as follows: WAF1, pCEP-WAF1 (6); cyclin A, Rc/CMV-cycA (15); cyclin D1, Rc/CMV-cycD1 (27); cyclin D2, Rc/CMV-cycD2 (27); and cyclin E, Rc/CMV-cycE (15). The expression vectors were CMV.p21 (encoding the wild-type protein) and CMV.p21N and CMV.p21C (encoding the N- and C-terminal domains, respectively, of WAF1) (3). The small-t expression vectors (33) were pCMV5-st (encoding the wild-type protein) and pCMV5-stΔ#3 (encoding the small-t antigen [small t] with a deletion in the PP2A-binding domain).

Affinity cell sorting and flow cytometry. Cells cotransfected with pCMV-IL2R (11) and a nonselected test plasmid were purified by magnetic affinity cell sorting after expression and chase periods (see below). Cells were washed with phosphate-buffered saline (PBS) and incubated for 30 min with anti-Tac monoclonal antibody-coated magnetic particles in medium S (PBS containing 4 mM EGTA, 1 mM $MgCl_2$, 10 mM HEPES [pH 8.0], chondroitin sulfate [100 μg/ml], gelatin [1 mg/ml], nonfat dry milk [8 mg/ml], and bovine serum albumin [10 μg/ml]). After adsorption of magnetic particles to Tac antigen-positive transfectants, the cell monolayer was washed with PBS and cells were removed by brief exposure to trypsin followed by addition of excess soybean trypsin inhibitor. Suspended cells were subjected to three cycles of magnetic separation in medium S followed by a wash in PBS. For Western blotting, cells were processed directly (see below). For double-parameter flow cytometry (DPFC), cells were kept at −70°C before preparation of nuclei in citrate buffer (36). Nuclei were fixed with 70% ethanol, denatured with 2 N HCl, and stained with antibromodeoxyuridine (anti-BrdU) (Becton-Dickinson) and propidium iodide. Flow cytometry was performed on a Becton-Dickinson FACScan flow cytometer with a doublet discrimination module. In a typical experiment, 3×10^3 to 5×10^3 events were collected and analyzed by using Lysis II software (Becton-Dickinson). As there was a variation from experiment to experiment in the absolute values of DNA staining, the gating of S-phase cells stained or unstained with BrdU was performed for each

experiment independently, and the percentages of cells gated were obtained by using Lysis II software. An example of such gating is shown in Fig. 2.

BrdU pulse-chase assay. Asynchronous SAOS-2 cells (1.5×10^6/T-25 flask) were labeled with 10 μmol of BrdU for 30 min and electroporated with pCMV-IL2R (1 μg) (11) with or without additional expression plasmids (5 μg each). pCMV.3, which has a polylinker downstream from the cytomegalovirus promoter, was included when appropriate to equalize the total DNA used in transfections. After electroporation, cells were plated in DMEM with 10% FBS and 2 mM thymidine to maintain BrdU-positive cells in S phase and to allow accumulation of expressed proteins. Expression from transfected plasmids was enhanced by addition of 5 mM sodium butyrate (12). At 16 h after transfection, cells were washed to remove thymidine and put in chase medium (DMEM, 10% FBS, 5 mM sodium butyrate, 10 mM deoxycytidine). After a chase period of 10 h, the transfected subpopulation was sorted and nuclei were analyzed by DPFC as described previously (23). The experiments were performed two or three times, with similar results.

Western immunoblot analysis. The extracts from the sorted cells were subjected to Western blot analysis as previously described (35). Antibodies obtained from PharMingen included anti-Sdi1 (catalog no. 15091), anti-PCNA (catalog no. 32551), anti-cyclin A (catalog no. 14531), anti-cyclin D (catalog no. 14841), anti-cyclin E (catalog no. 14591), anti-simian virus 40 (SV40) T (catalog no. 14121), and anti-Rb (catalog no. 14001). Polyclonal antibodies against full-length p21 were obtained from Santa Cruz (catalog no. sc-756).

The extracts applied to each lane were derived from equal numbers of cells. In order to analyze the amount or state of a particular protein in S phase, the cells were first blocked by thymidine for 24 h, transfected with expression plasmids, and incubated for another 16 h in thymidine. The extracts from the sorted cells were analyzed by Western blotting. In this experimental setting, only S-phase cells are present in the analyzed sample, which was confirmed by flow cytometry with propidium iodide.

RESULTS

As a means to assess the involvement of CDK and CDK inhibitor in controlling S phase as opposed to other phases of the cell cycle, the basic parameter on which we focused was the fraction of cells that moved from intra-S phase to completion of DNA replication (i.e., to a G_2-M DNA content) during a given time interval. This was accomplished as follows (Fig. 1): cells were labeled briefly with BrdU, held in S phase during transfection and an expression period, and then allowed to continue through the cycle under conditions which prevented further BrdU incorporation. Transit through S phase was followed by DPFC. Since the BrdU-labeled cells entered S phase prior to cell cycle manipulation, this experimental setting allowed us to neglect the effect(s) of a given intervention on G_1-S transit.

WAF1 retards S-phase progression. To ascertain whether WAF1 overexpression can delay the completion of S phase, SAOS-2 cells were electroporated with a cell surface marker plasmid together with a cytomegalovirus promoter-driven expression vector encoding WAF1 (Fig. 1). After BrdU labeling and expression and chase periods, the successfully transfected subpopulation was purified by affinity sorting and subjected to flow cytometric analysis (23, 29). Control BrdU-labeled cells were observed to exit S phase and accumulate in either G_2-M or G_1, while new unlabeled cells entered S phase (Fig. 2A). In the WAF1-transfected culture, the fraction of BrdU-labeled cells which remained in S phase increased more than twofold. Also, consistent with the notion that WAF1 blocks G_1-S transit (6, 13), no new cells entered S phase in the latter culture.

WAF1 is a well-characterized inhibitor of CDKs (6, 13, 38). We next confirmed that WAF1 is overexpressed in the transfected cells (Fig. 3B) in amounts sufficient to inhibit CDK activity. This was demonstrated by cotransfection of WAF1 with a plasmid encoding the retinoblastoma gene product, pRb, a major physiological target of CDKs (Fig. 3A) (also see the description of conditions in Materials and Methods). Suppression of pRb hyperphosphorylation in S-phase SAOS-2 cells was seen, consistent with exogenous WAF1 causing a substantial decrease in CDK activity.

We conclude that the upregulation of WAF1 expression, in addition to its previously reported effect on the G_1-S transition, has an inhibitory effect on the progression of cells through S phase.

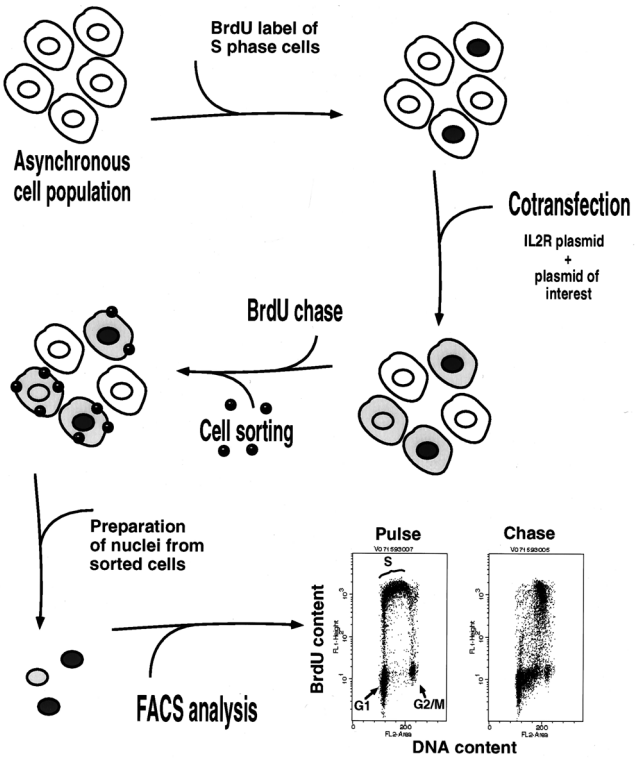

FIG. 1. Schematic diagram of experimental procedure. Asynchronous cells are pulse labeled with BrdU (see Materials and Methods) and then cotransfected with WAF1-expressing plasmid and the sorting vector pCMV-IL2R expressing surface marker. After transfection, BrdU-labeled cells are held in S phase for 16 h by thymidine block and then chased for 10 h. The transfected cells, expressing surface marker IL2R, are then affinity sorted by using magnetic beads coupled with anti-IL2R antibodies. Nuclei from the sorted cells are analyzed by double-parameter (on a fluorescence-activated cell sorter [FACS]). The progression of BrdU-labeled cells through the S phase is observed by comparing the DNA content (propidium iodide staining) distribution of chased versus pulse-labeled sample.

WAF1 inhibition cannot be reversed by PCNA overexpression. WAF1 could delay S-phase progression either by down-regulation of CDK activity or by interaction with the replication-repair factor PCNA. The inhibitory effect of WAF1 in an in vitro DNA replication system can be reversed by addition of PCNA; moreover, these proteins have been shown to interact directly (10, 37). To determine whether the effect of WAF1 in our system could be suppressed by additional PCNA, WAF1 and PCNA expression plasmids were cotransfected. No change was observed in terms of cell cycle distribution relative to that seen with cells transfected with WAF1 alone (Fig. 2A, compare panels 3 and 4; Table 1). Nor did overexpression of PCNA alone have any effect on the S-phase progression (data not shown). The failure of PCNA overexpression to reverse WAF1-inhibitory action was not due to inability of ectopically expressed PCNA to bind WAF1, as it could be coprecipitated with WAF1 (Fig. 3E). Moreover, it was not due to an inadequate increment in PCNA levels, since the amount of PCNA in pCMV-PCNA-transfected cells, as measured by quantitative Western analysis, exceeded by more than fourfold the amount of WAF1 (Fig. 3D). Since cells were held in S phase to evaluate relative WAF1 and PCNA levels, it is further evident that WAF1 is not degraded to allow S-phase progression.

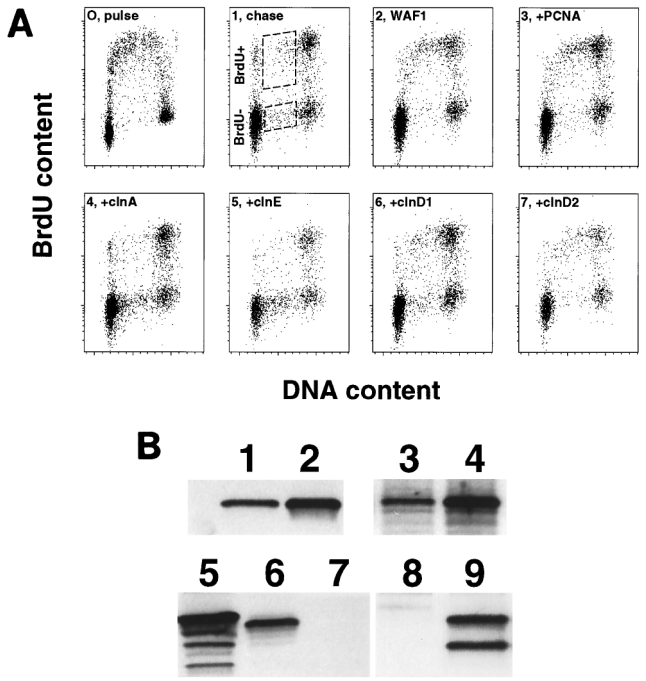

FIG. 2. Expression of WAF1 can block SAOS-2 cells in S phase; overexpression of cyclins but not PCNA is sufficient to overcome WAF1 inhibition. (A) SAOS-2 cells were labeled with BrdU and electroporated with pCMV-IL2R with or without additional expression plasmids. After electroporation, cells were plated in the presence of 2 mM thymidine to maintain BrdU-positive cells in S phase and to allow accumulation of expressed proteins. On the following day, cells were washed to remove thymidine and put in chase medium. After the chase, the transfected subpopulation was sorted and nuclei were analyzed by fluorescence-activated cell sorting. Panels: 0, BrdU-unstained pulse-labeled cells; 1, control; 2, WAF1; 3, WAF1 plus PCNA; 4, WAF1 plus cyclin A; 5, WAF1 plus cyclin E; 6, WAF1 plus cyclin D1; 7, WAF1 plus cyclin D2. The gating of the cells with S-phase content and no BrdU staining (G_1 cells entering the S phase during the chase) and the cells with S-phase content that stained positive for BrdU (arrested in S phase) are indicated in panel 1. (B) Western blot analysis showing levels of PCNA or cyclins following transfection with sorting vector plus PCNA or cyclin expression plasmids as indicated. Lanes: 1, 3, 7, and 8, controls; 2, PCNA; 4, cyclin A; 5, cyclin D1; 6, cyclin D2; 9, cyclin E. Probes were antibodies against the following: PCNA (lanes 1 and 2), cyclin A (lanes 3 and 4), cyclin D (lanes 5 to 7), and cyclin E (lanes 8 and 9).

Overexpression of cyclins can reverse the WAF1 effect. We next tested whether the effect of WAF1 could be suppressed when this protein was coexpressed with components of the cyclin or CDK machinery. As seen in Fig. 2A and Table 1, cyclins A and E reversed the inhibitory action of WAF1 almost completely, while cyclin D2 had a moderate effect and cyclin D1 was inactive. CDK2 and CDK4 transfection partially reversed WAF1 activity (not shown). The expression of cyclins alone did not accelerate S-phase progression in SAOS-2 cells but did stimulate the G_1-S transition (not shown).

Control experiments ruled out several indirect mechanisms by which cyclins might have been acting. None of the cyclins decreased the amount of WAF1 in transfected cells (Fig. 3B), excluding the possibility that the observed suppression of WAF1 effects was due to an effect on the level of WAF1 expression (coexpression of D1 cyclin, surprisingly, increased the level of WAF1, so we cannot comment on the ability of this particular cyclin to antagonize WAF1 function). Comparison of PCNA expression in the same samples (Fig. 3C) shows that the level of this protein was elevated only in the PCNA plasmid-transfected cells. This excluded the possibility that CDKs antagonize WAF1 indirectly via the induction of elevated PCNA levels. Consistent with their role in CDK activation, the cotransfection of cyclins could suppress the CDK-inhibitory effect of WAF1 on pRb hyperphosphorylation (Fig. 3A).

The CDK binding domain of WAF1 has a much stronger effect on S-phase progression than the PCNA binding domain. The preceding data are most compatible with the notion that inhibition of CDK activity is primarily responsible for the S-phase effect of WAF1. In agreement with this conclusion, expression of the CDK binding N-terminal domain of WAF1 (3) had the same effect on S-phase progression as expression of the full-length protein (Fig. 4A and Table 2), while the PCNA binding C-terminal domain exhibited only modest activity (Fig. 4A and Table 2), in spite of higher levels of expression (Fig. 4B; see also Discussion).

These experiments were repeated with U2OS, another osteosarcoma cell line. In contrast to SAOS-2 cells, U2OS cells carry functionally active pRb and p53 proteins. As can be seen from Table 2, the effects of full-length WAF1, as well as of the N-terminal and C-terminal derivatives, were similar in the SAOS-2 and U2OS lines. This confirms that the CDK-dependent inhibition of S-phase progression by WAF1 is not limited to a particular cell type.

WAF1 effect is antagonized by SV40 small t. Interestingly, we observed that coexpression of SV40 small t almost completely reversed the WAF1-mediated inhibition of S-phase progression (Fig. 5A and Table 1). Consistent with a functional, although presumably indirect, interaction between WAF1 and small t, small t expressed alone did not accelerate S-phase progression (data not shown). A trivial explanation for the antagonism between small t and WAF1 could be ruled out, since increased levels of endogenous cyclins A and E (Fig. 5B and data not shown) or PCNA (Fig. 5B) were not evident in small t-expressing cells. Small t mimicked some, but not all, effects of cyclins A and E in reversing WAF1 activity. In contrast to the cyclin A or E cotransfection, small t was not able to overcome the ability of WAF1 to retard G_1-S transit (Fig. 5A and unpublished results). In accord with this, WAF1 caused a decrease in the number of BrdU-negative cells with an S-phase DNA content both in the presence and in the absence of small t. Further, cotransfection with small t (or with cyclin E) failed to regenerate BrdU-labeled cells with a G_1 DNA content, despite facilitating S-phase progression in the presence of WAF1. The latter result was revealed in a separate study to be due to inhibition of G_2-M progression in SAOS-2 cells by small t or cyclin E alone (unpublished results).

To date, the only known activity of SV40 small t is binding to and blocking the function of the Ser/Thr phosphatase PP2A; furthermore, PP2A has been implicated in cell cycle control by small t (21, 33). As can be seen in Fig. 5A and Table 1, a small t deletion mutant lacking the PP2A-binding domain (stΔ) failed to overcome WAF1 inhibition, even though it was expressed in amounts exceeding the wild-type protein. While indirect mechanisms cannot be excluded, the ability of small t to reverse the WAF1 effect thus seems most likely to depend on its interaction with the protein phosphatase PP2A or similar factors.

DISCUSSION

We have succeeded in dissociating S-phase effects of CDKs and the CDK inhibitor WAF1 from the G_1-S and G_2-M regulatory properties of these proteins. This was accomplished by labeling cells with BrdU prior to transfection, allowing subsequent completion of replicative DNA synthesis, and analyzing the results by a combination of affinity cell sorting of transiently transfected cells and DPFC. The results presented here

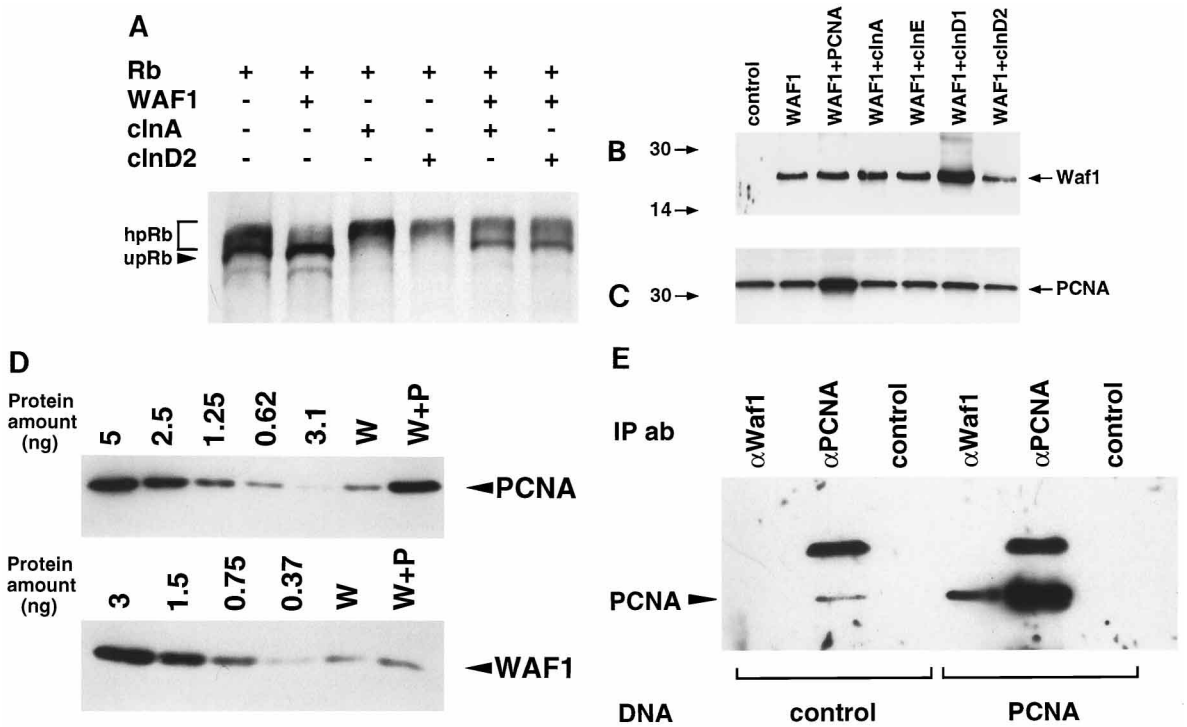

FIG. 3. Analysis of PCNA and cyclin overexpression in S-phase SAOS-2 cells. (A) Western blot analysis showing the state of pRb cotransfected in S-phase SAOS-2 cells with WAF1 and/or cyclin A (clnA) or cyclin D2 (clnD2). The cells were treated as described in Materials and Methods. hpRb and upRb, hyperphosphorylated and underphosphorylated forms of pRb, respectively. (B and C) Western blot analysis showing levels of WAF1 (B) and PCNA (C), following transfection of S-phase SAOS-2 cells with sorting vector plus WAF1 and PCNA or cyclin expression plasmids. The positions of marker proteins are indicated on the left (in kilodaltons). (D) Western blot analysis comparing the absolute amounts of PCNA and WAF1 in pCMV.PCNA- and pCMV.WAF1-cotransfected S-phase SAOS-2 cells. The amounts of protein WAF1 and PCNA (both generous gifts of P. O'Connor) are indicated above the lanes. W, 5 μl of extract from transfection with WAF1 alone; W+P, 5 μl of extract from cotransfection of WAF1 and PCNA. (E) Coimmunoprecipitation experiment demonstrating that overexpressed PCNA can bind to WAF1. S-phase SAOS-2 cells were transfected with control DNA (first three lanes) or pCMV-PCNA (last three lanes). Extract from the sorted cells was processed for immunoprecipitation (IP) by polyclonal anti-Waf1 (αWaf1), monoclonal anti-PCNA (αPCNA), or polyclonal mock (control) antibodies (ab). The immunoprecipitates were then analyzed by Western blotting using anti-PCNA antibodies. The band corresponding to PCNA is indicated. The extra band above the PCNA band in the αPCNA precipitates corresponds to mouse immunoglobulin G.

strongly support the notion that CDK activity is involved in the regulation of S-phase progression in mammalian cells.

Our conclusions are in agreement with genetic data from *Saccharomyces cerevisiae* (7, 31) that point to a role for cyclins clb5 and clb6 in the completion of S phase. Data from in vitro studies with *Xenopus* eggs (34) are also consistent, in that inhibition of CDK2 activity has been shown to block initiation of DNA replication in a manner which can be restored by addition of cyclin A or E. The identities of CDK substrates that must be phosphorylated in order to maintain normal S-phase progression are not known; however, the fact that inhibition of CDKs in vitro did not affect replication of single-stranded DNA in the *Xenopus* egg extract suggests that the mechanism involves the initiation and/or elongation phase of double-stranded DNA replication (3, 34).

Complementary to the CDK and WAF1 results is evidence that SV40 small t can affect S-phase progression. Since the only known target of small t is the Ser/Thr phosphatase PP2A, this result suggests a potential indirect role of PP2A in S-phase progression. Evidence for involvement of this enzyme in the regulation of G_1-S transit has been reported earlier, based, in particular, on the ability of SV40 small t to bind and inactivate PP2A in vitro (28, 30, 39). Small t was demonstrated to cooperate with large T antigen in transformation of rodent cells under restrictive growth conditions (2, 20) as well as in stimulation of DNA synthesis and hyperphosphorylation of the *retinoblastoma* gene product in human diploid fibroblasts (23).

TABLE 1. Effect of transfection with WAF1 and cyclins or PCNA on distribution of BrdU-labeled cells after chase

Expt and DNA	No. of cells in S phase	Total no. of cells	% Cells in S phase
Expt 1			
Control	118	967	12
pCMV.Waf1	217	795	27
pCMV.Waf1 plus:			
pCMV.clnD1	125	485	27
pCMV.clnD2	171	921	19
pCMV.clnA	72	940	7.4
pCMV.clnE	42	433	9.7
pCMV.small twt	95	632	15
pCMV.small tΔ	243	970	25
pCMV-PCNA	234	964	24
Expt 2			
Control	158	1,576	10
pCMV.Waf1	350	1,460	24
pCMV.Waf1 plus:			
pCMV.clnA	123	1,366	9
pCMV.clnE	174	1,743	10
pCMV-PCNA	363	1,580	23
pCMV.small twt	167	1,286	13
pCMV.small tΔ	327	1,364	24
pCMV.clnA alone	116	1,444	8
pCMV.clnE alone	169	1,523	11
pCMV-PCNA alone	143	1,298	11

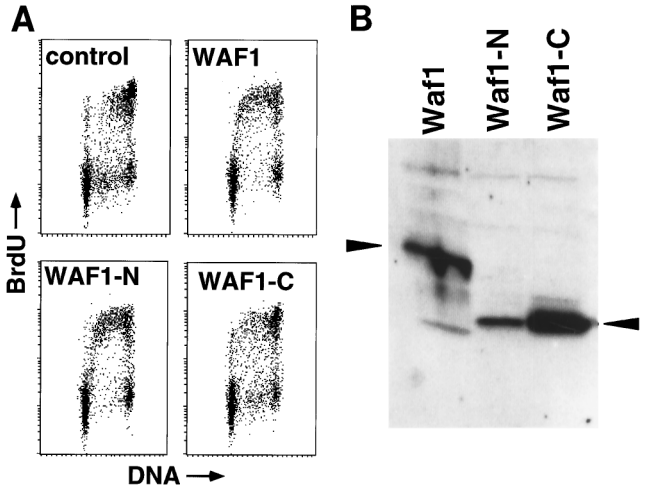

FIG. 4. CDK-interacting domain of WAF1 has the same effect on S-phase progression as the wild-type protein. (A) DPFC analysis of SAOS-2 cells subjected to the BrdU pulse-chase protocol and transfection with sorting vector plus WAF1 derivatives. WAF1-N, N-terminal part of WAF1; WAF1-C, C-terminal part of WAF1. (B) Western blot analysis showing levels of expression of the full-length protein and its truncated versions following transfection with the following corresponding expression plasmids: p21 (WAF1), p21N (WAF1-N), p21C (WAF1-C). Corresponding bands are indicated by arrowheads.

Recently, small t was reported to stimulate the MAP kinase pathway indirectly by interacting with PP2A (33), and we have observed that small t alone is able to accelerate G_1-S transit in several cell types (unpublished results). In view of the central role of protein kinases in the regulation of G_1-S transit, an appealing view is that their activity is balanced by the complementary function of the protein phosphatases. It is tempting in turn to speculate that a similar balance might be found in the case of S-phase progression. Supporting such a balance in the regulation of S phase, the PP2A binding activity of small t is also able to revert the S-phase arrest induced by pRb (unpublished results).

A further issue to be considered is how S-phase progression can be regulated in case of exogenous injury that results in DNA damage. UV irradiation, for example, induces arrest in S phase as well as blocks in G_1 and G_2 (18). The involvement of WAF1 in DNA damage-inducible G_1 arrest mediated by p53 (6) suggests that this protein is likewise a strong candidate to play a role in S-phase arrest. To the extent that this is the case, the biologically relevant question arises as to whether the primary target for WAF1-mediated intra-S-phase arrest is PCNA

TABLE 2. Effect of CDK or PCNA binding domains of WAF1 on S-phase progression of BrdU-labeled cells

Cell line and DNA	No. of cells in S phase	Total no. of cells	% Cells in S phase
SAOS-2			
Control	311	1,635	19
pCMV.p21wt	784	1,782	44
pCMV.p21N	623	1,355	46
pCMV.p21C	457	1,634	28
U2OS			
Control	123	1,122	11
pCMV.p21wt	355	986	36
pCMV.p21N	474	1,216	39
pCMV.p21C	211	1,174	18

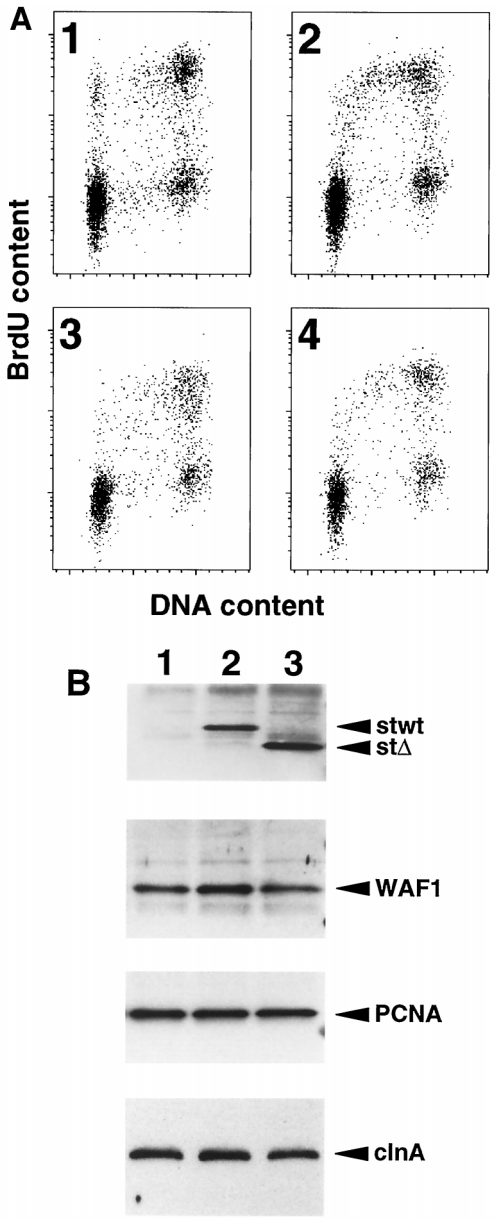

FIG. 5. The ability of SV40 small t to overcome the effect of WAF1 is dependent on its PP2A binding domain. (A) DPFC analysis of SAOS-2 cells subjected to the BrdU pulse-chase protocol and transfection with sorting vector plus WAF1 and SV40 small t expression plasmids as indicated. Panels: 1, control; 2, WAF1; 3, WAF1 plus small t (wild type); 4, WAF1 plus stΔ (with the PP2A-binding domain deleted). (B) Western blot analysis showing levels of small t, WAF1, PCNA, and cyclin A (clnA) following transfection with sorting vector plus WAF1 and small t expression plasmids as indicated. Lanes: 1, WAF1 alone; 2, WAF1 plus small t (wild type) (stwt); 3, WAF1 plus stΔ (with the PP2A-binding domain deleted). Corresponding proteins are indicated by arrowheads.

or cyclin-CDK complexes. DNA replication in vitro can be directly inhibited by WAF1 through the PCNA binding, and it has been suggested that S-phase progression in vivo can be inhibited by this mechanism (10, 37). Evidence presented here, however, implicates CDKs as the first targets of induced WAF1. The reason for this may be a relative abundance of PCNA compared to components of the CDK machinery, as has been suggested for the *Xenopus* egg system (3); accordingly, relatively high levels of WAF1 expression might be necessary

to observe a direct block of DNA replication due to PCNA titration.

While CDKs are evidently the primary targets of WAF1 induction, we also noticed a weaker effect of the C-terminal domain of WAF1 on S-phase progression. The latter result is in agreement with earlier data (19); however, it must be stressed that the methodology used in that study did not allow one to discern which part of the cell cycle was affected, while the approach described here has been designed specifically to dissect the complex effects of CDKs and their inhibitors on different stages of the cell cycle. According to Adams et al. and Chen et al. (1, 4), the C terminus of WAF1 has a weak cyclin-CDK binding motif; thus, the effect of this WAF1 domain may be partially due to CDK inhibition as well. In keeping with this possibility, both A and E cyclins and PCNA could partially overcome the effect of the C terminus (23a).

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