v-Abl Protein-tyrosine Kinase Up-regulates p21<sup>WAF-1</sup> in Cell Cycle Arrested and Proliferating Myeloid Cells*

Sophia J. Khanna‡§, Robin Brown¶, Anthony D. Whetton†, Kathryn L. Ball**, and Caroline Dive‡ †‡

From the ‡Cancer Research Campaign Molecular Pharmacology Group, School of Biological Sciences, University of Manchester, G38 Stopford Building, Oxford Road, Manchester M13 9PT, ¶Glaxo Wellcome Research & Development, Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, the Leukaemia Research Fund Cellular Development Unit, Department of Biomolecular Sciences, University of Manchester Institute of Science and Technology, Sackville Street, Manchester M60 1QD, and the **Cancer Research Campaign Laboratory, Biomedical Research Centre, Ninewells Medical School, University of Dundee, Dundee DD1 9YJ, United Kingdom

v-Abl protein-tyrosine kinase (PTK) promotes cell survival without cell proliferation in interleukin (IL)-3-deprived IC.DP premast cells (1). We now show that in these conditions v-Abl PTK transcriptionally up-regulated the cyclin-dependent kinase inhibitor (CDKI) p21<sup>WAF-1</sup> and inhibited CDK2 and CDK4. When readdition of IL-3 stimulated cell proliferation, p21<sup>WAF-1</sup> was inactivated as a CDKI despite maintenance of elevated protein level. p21<sup>WAF-1</sup> was also up-regulated yet was nonfunctional as a CDKI when v-Abl PTK was activated in cells maintained in IL-3, but this occurred without increased p21<sup>WAF-1</sup> transcription. Using a C-terminal epitope-specific p21<sup>WAF-1</sup> antibody, v-Abl PTK-mediated increase in p21<sup>WAF-1</sup> could be detected in intact cells only in the presence of IL-3. This indicated different binding partners of p21<sup>WAF-1</sup> and/or protein conformation in nondividing or proliferating cells, respectively. The binding of CDK2, CDK4, or proliferating cell nuclear antigen to p21<sup>WAF-1</sup> and its subcellular localization were unchanged in the presence or absence of IL-3. However, two-dimensional analysis revealed different forms of up-regulated p21<sup>WAF-1</sup> in IL-3-deprived, nondividing cells compared with IL-3-stimulated proliferating cells. These data demonstrate that elevation of the CDKI p21<sup>WAF-1</sup> is not always sufficient for cell cycle arrest and indicate an IL-3-sensitive pathway for the inactivation of p21<sup>WAF-1</sup> function as a CDKI.

One mechanism by which cell cycle progression is regulated is via the balance between cyclin-dependent kinase (CDK) activation and inhibition (2). The CDK inhibitor (CDKI) p21<sup>WAF-1</sup>, the prototype of the Cip/Kip family of CDKIs, was initially identified in a communoprecipitate with cyclin D (3, 4). p21<sup>WAF-1</sup> was shown to bind to and inhibit cyclin-CDK complexes (5), incorporating PCNA into a quaternary complex (3) and thereby regulating the cell cycle machinery. Initial observations by Xiong et al. (6) described the primary role for p21<sup>WAF-1</sup> as an inhibitor of CDK activity. However, subsequent investigations suggested that p21<sup>WAF-1</sup> may also function to assemble specifically CDK4-cyclin D complexes and to target them to the nucleus (7). This has recently been substantiated in murine fibroblasts lacking p21<sup>WAF-1</sup>, wherein CDK4 and cyclin D cannot associate (8). The generally accepted theory surrounding p21<sup>WAF-1</sup> function therefore suggests that p21<sup>WAF-1</sup> is involved in the assembly of CDK4 complexes but inhibits the activity of CDK2. However, p21<sup>WAF-1</sup>-nullizygous mice are developmentally normal (9) and display normal CDK4 activity, thereby questioning the physiological role for p21<sup>WAF-1</sup> in the assembly specifically of CDK4.

The putative C-terminal nuclear localization signal in p21<sup>WAF-1</sup> may help target the quaternary complex to the nucleus where it may exert its effect on CDKs. At the onset of mitosis, p21<sup>WAF-1</sup> up-regulation caused the nuclear accumulation of CDK4 associated with cyclins A and B1 (10). p21<sup>WAF-1</sup> also directs PCNA to the replicative machinery of the cell (11), and cell cycle arrest caused by p21<sup>WAF-1</sup> involves inhibition of PCNA function(s). p21<sup>WAF-1</sup> is required for cell cycle arrest at G<sub>1</sub> phase of the cell cycle; HCT116 colon carcinoma cells containing wild-type p21<sup>WAF-1</sup> undergo growth arrest after DNA damage, whereas their p21<sup>WAF-1</sup>-null counterparts do not (12). Moreover, cells derived from p21<sup>WAF-1</sup>-null mice have defective G<sub>1</sub> checkpoints (9), p21<sup>WAF-1</sup> also functions at other phases of the cell cycle. During S phase p21<sup>WAF-1</sup> prevented replication of DNA possibly because of the inhibitory effect on PCNA (11) or because of p21<sup>WAF-1</sup> mediated inhibition of CDK activity (14). p21<sup>WAF-1</sup> overexpression was linked with G<sub>1</sub> and G<sub>2</sub> arrest and prevented entry into S phase, although no delay in S phase progression was observed (15). Conversely, down-regulation of p21<sup>WAF-1</sup> allowed DNA synthesis and entry into mitosis, thereby reversing growth arrest (16).

We noted previously that the activation of a temperature-sensitive mutant of v-Abl protein-tyrosine kinase (PTK), a leukemogenic oncogene promoted cell survival without cell proliferation after the withdrawal of IL-3 (1). Cell survival was associated with the up-regulation of Bcl-x<sub>L</sub> (17). Down-regulation of Bcl-x<sub>L</sub> using an antisense approach restored an apoptotic response to IL-3 deprivation with v-Abl PTK active. However, the mechanism whereby v-Abl PTK promoted growth

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a Biotechnology and Biological Sciences Research Council postgraduate studentship with GlaxoWellcome.

¶ Supported by the Lister Institute of Preventive Medicine. To whom correspondence should be addressed. Tel.: 44-161-275-5495; Fax: 44-161-275-5600; E-mail: cdive@man.ac.uk.

1 The abbreviations used are: CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; PCNA, proliferating cell nuclear antigen; PTK, protein-tyrosine kinase; IL, interleukin; BrU, bromodeoxyuridine; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription; PCR, polymerase chain reaction; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

2 Q. Chen and C. Dive, unpublished results.
arrest remained unknown. Here we describe the transcriptional up-regulation of p21\textsuperscript{WAF-1} 3 h after IL-3 withdrawal in cells with active v-Abl PTK. We also report that readdition of IL-3 to cells with v-Abl PTK active prevented the inhibition of cells with active v-Abl PTK. We also report that readdition of IL-3 to cells with v-Abl PTK active prevented the inhibition of CDK2 and CDK4, allowing proliferation despite the maintained high levels of p21\textsuperscript{WAF-1} expression.

**EXPERIMENTAL PROCEDURES**

**Reagents**—For Western blot analyses, p21\textsuperscript{WAF-1} was detected with either monoclonal anti-p21\textsuperscript{WAF-1} Ab-4 or polyclonal anti-p21\textsuperscript{WAF-1} Ab-5 (Calbiochem, Cambridge, MA). PCNA was detected with monoclonal antibody PC10, CDK2 with polyclonal antibody C22, and CDK4 with polyclonal antibody M2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Actin was detected with monoclonal anti-actin antibody (Sigma). All primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark) and visualized using Enhanced Chemiluminescence (PerkinElmer Life Sciences). For flow cytometric analyses, p21\textsuperscript{WAF-1} was detected using monoclonal anti-p21\textsuperscript{WAF-1} antibodies Ab-4, Ab-6 (Calbiochem), or WA-1 (kindly provided by Dr. Borek Vojlesek). The irrelevant antibody control was IgG\textsubscript{1} mouse anti-Aspergillus niger glucose oxidase antibody. Primary antibodies were detected using a fluorescein isothiocyanate-conjugated secondary antibody (Dako). For confocal microscopy, monoclonal anti-p21\textsuperscript{WAF-1} antibodies SX118 (Calbiochem) or polyclonal anti-p21\textsuperscript{WAF-1} was used followed by Alexa fluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR). Unless otherwise stated, all other reagents were obtained from Sigma.

**Cell Culture**—The IC.DP cell line was derived from IL-3-dependent murine mast progenitor cell line IC2.9 by stable transfection of a temperature-sensitive mutant of the v-Abl PTK (18). Cells were routinely maintained in Fischer’s medium supplemented with horse serum (10% v/v) (both from Life Technologies, Inc.) and IL-3 conditioned medium (3% v/v) (19). For IL-3 withdrawal experiments, v-Abl PTK was activated or inactivated by incubation of cells at 32 or 39 °C respectively, for 2 h prior to washing cells in Fischer’s medium (1). Cells were then cultured for up to 96 h at either temperature. Where appropriate, IL-3 was restored to the culture medium of IC.DP cells with v-Abl PTK active for 48 h, after the initial IL-3 withdrawal, or cells were maintained in the presence of IL-3 throughout the experiment. Cells were treated with actinomycin D (1 \mu g/ml), cycloheximide (10 \mu g/ml), or etoposide (10 \mu M) as appropriate. Parental IC2.9 cells were used as a control for the effect of culture temperature.

**RESULTS**

**Fig. 1.** Cell death kinetics following IL-3 withdrawal from IC.DP and IC2.9 murine premast cells. Viable cell number was assessed following the withdrawal of IL-3. IC.DP at 32 °C (v-Abl PTK active; □); IC.DP at 39 °C (v-Abl PTK inactive; △); IC2.9 at 32 °C (no v-Abl PTK; ■); IC2.9 at 39 °C (no v-Abl PTK; ◇).

**Table 1**

| Time after IL-3 withdrawal | G\textsubscript{1} mean ± S.E. (n = 3) | S mean ± S.E. (n = 3) | G\textsubscript{2}/M mean ± S.E. (n = 3) |
|---------------------------|-----------------------------------|----------------------|-----------------------------------|
| 0 h                       | 76 ± 2                            | 8 ± 1                | 15 ± 1                            |
| 24 h                      | 85 ± 0                            | 6 ± 2                | 8 ± 2                             |
| 48 h                      | 82 ± 1                            | 5 ± 1                | 13 ± 2                            |
| 72 h                      | 84 ± 1                            | 5 ± 1                | 10 ± 1                            |

**Fig. 2.** Up-regulation of p21\textsuperscript{WAF-1} by v-Abl PTK activation after withdrawal of IL-3. Western blot analysis of p21\textsuperscript{WAF-1} protein expression following IL-3 withdrawal from IC.DP cells (A) and IC2.9 cells (B). Also indicated is the percentage of cell death occurring in the cell samples from which the lysates were made. The data shown are representative of three independent repeat experiments.

**Cell Cycle Analysis by Flow Cytometry**—Cells were fixed in ice-cold methanol (70% v/v) and incubated with propidium iodide (Molecular Probes; 10 \mu g/ml) for 5 min prior to analysis of DNA content by flow cytometry. Red fluorescence (DNA-bound propidium, linear scale, 630 ± 22 nm) was analyzed by a Becton Dickinson FACSVantage cytometer (Becton Dickinson, San Jose, CA) using the 488-nm line of the Enterprise laser (Coherent, Palo Alto, CA) set to excite at 250 mW. Cell debris was excluded by electronic gating of forward and orthogonal light scatter profiles, prior to analysis of DNA content.

**Immunostaining for p21\textsuperscript{WAF-1} by Flow Cytometry and Confocal Microscopy**—For flow cytometric analyses of p21\textsuperscript{WAF-1}, cells were harvested and fixed in formaldehyde (1% v/v in phosphate-buffered saline). Immunostaining for p21\textsuperscript{WAF-1} was carried out using a panel of monoclonal anti-p21\textsuperscript{WAF-1} antibodies (Ab-4, Ab-6, and WA-1). The majority of experiments were conducted using WA-1 (1:100 in phosphate-buffered saline containing 500 \mu g/ml digitonin) and secondary antibody goat anti-mouse conjugated to fluorescein isothiocyanate (1:40). Cell debris was excluded, and forward and orthogonal light scatter were measured simultaneously with green fluorescence (fluorescein isothiocyanate-conjugated antibody; 530 ± 30 nm, log scale) as described above. Data were analyzed using CellQuest and CellFit BD software. Statistical analysis was by the two-tailed Student’s t test at the p = 0.05 level of significance. Analysis was performed using the Microsoft Excel package (Microsoft Corporation, Seattle, WA).

For confocal microscopy, cells were harvested and cytosplasm on glass slides before fixing in ice-cold methanol/acetic acid (1:1 v/v) for 5 min. Cells were incubated with blocking/permeabilization buffer (0.15 M NaCl, 5 mM KCl, 0.3 mM Na\textsubscript{2}HPO\textsubscript{4}, and 25 mM Trizma base) containing bovine serum albumin (0.1% w/v), Triton X (0.1% v/v), and fetal calf serum (1% v/v) for 30 min. p21\textsuperscript{WAF-1} was detected using SX118 at 1 \mu g/ml or polyclonal anti-p21\textsuperscript{WAF-1} at 5 \mu g/ml in diluent (blocking buffer containing bovine serum albumin (0.1% w/v), Triton X (0.05% v/v), and fetal calf serum (1% v/v)) for 1 h, followed by anti-mouse 488 Alexa antibody (1:100 in diluent) for 30 min. Nuclei were stained with propidium iodide (1 \mu g/ml containing 100 \mu g/ml RNase) by incubation at 37 °C for 30 min. Fluorescence was detected with excitation wavelengths of 488 or 568 nm using a Leica TCS-4D confocal microscope.

**Analysis of S Phase Traverse by BrdU Incorporation**—Cells were pulse-labeled with BrdU (100 \mu M) for 1 h at 32 °C and fixed in 1 ml formaldehyde (1% w/v in phosphate-buffered saline). DNA was partially denatured by treatment with 1 ml of 2 N HCl for 30 min at room temperature. Incorporated BrdU was detected by flow cytometry after incubation of cells with monoclonal anti-BrdU antibody (1:20 in phosphate-buffered saline containing 500 \mu g/ml digitonin) for 1 h at room
temperature followed by incubation with goat anti-mouse fluorescein isothiocyanate secondary antibody (1:40) for 30 min. DNA was counterstained with propidium iodide, and red fluorescence was analyzed as described above.

**Western Blotting**—Western blotting was carried out according to a standard protocol (20). In brief, 30 μg of protein from cell lysates were subjected to SDS-PAGE on 14% gels and transferred onto polyvinylidene difluoride membranes. Immunodetection of p21WAF-1 was conducted using Ab-4 (1 μg/ml) and goat anti-mouse horseradish peroxidase (1:3000), followed by detection by enhanced chemiluminescence (see above). Equal protein loading was verified by measurement of actin levels. Protein level was assessed using an imaging densitometer and Molecular Analyst software (Bio-Rad).

**RT-PCR**—RNA was extracted using RNAzol (Biogenesis, Poole, UK) according to the manufacturer’s instructions. cDNA was synthesized using a reverse transcription-polymerase chain reaction kit (Stratagene Ltd, Cambridge, UK). PCR was then performed using murine specific p21WAF-1 primers (Sigma-Genosys Ltd., Pampisford, UK). Sense primer was 5'-CATTCAGAGCCACAGGCACC-3' and antisense primer and 5'-CTCCTGACCCACAGCAGAAG-3'.

**Assays for CDK2 and CDK4**—CDK2 and CDK4 activities were analyzed as previously described (21). In brief, 150 μg of protein from cell lysates was immunoprecipitated with 1 μg of polyclonal anti-CDK2 or CDK4 antibody. Immunoprecipitates were washed twice in 3kinase buffer: 100 mM Hepes, pH 7.4, 20 mM MgCl2, 5 mM EGTA, 20 mM β-glycerophosphate, 2 mM NaF, 2 mM dithiothreitol, and 20 μM cAMP-dependent kinase inhibitor protein kinase inhibitor. Kinase assays were prepared with 2× kinase buffer and calf thymus histone H1 (1 mg/ml). Reactions were started by addition of [32P]ATP (1:100 in 0.5 mM cold ATP in 10 mM Hepes, pH 7.4), incubated at 30 °C for 30 min and

**FIG. 3.** IL-3 promotes proliferation of IC.DP cells with maintained high levels of p21WAF-1. A, kinetics of the increase in viable cell number following readdition of IL-3 to cell cycle-arrested cells. Results shown are presented as the means ± S.E. (n = 3). B, S phase traverse in IL-3-restimulated IC.DP cells expressing active v-Abl PTK. IC.DP cells with active v-Abl PTK were deprived of IL-3 for 48 h before readdition of the cytokine. Data shown are representative of three independent repeat experiments. C, p21WAF-1 protein levels in IL-3-restimulated IC.DP cells containing active v-Abl PTK. Western blot analysis of p21WAF-1 protein expression following IL-3 readdition after 48 h IL-3 withdrawal from IC.DP cells with v-Abl PTK. The data shown are representative of three independent repeat experiments. D, p21WAF-1 protein expression in IC.DP cells with v-Abl PTK maintained in IL-3. Western blot analysis of p21WAF-1 protein expression in IC.DP cells with v-Abl PTK maintained in IL-3. The data shown are representative of three independent repeat experiments. E, panel v, shows the autoradiographs of 32P-labeled histone H1 incubated with either CDK2 or CDK4 immunoprecipitate. Data shown are representative of three independent repeat experiments.
terminated by heating to 95 °C in SDS-PAGE sample buffer, followed by SDS-PAGE analysis and autoradiography.

**Immunoprecipitation**—Cell lysates were prepared using Triton X-100 lysis buffer: 0.4% (v/v) Triton X-100, 150 mM KCl, 25 mM HEPES, pH 7.6, 5 mM dithiothreitol, 50 mM NaF, and protease inhibitor mixture. 150 μg of protein from cell lysates was preclaved with protein A-Sepharose (Amersham Pharmacia Biotech), followed by immunoprecipitation using polyclonal anti-p21\(^{WAF-1}\) Ab-5. Immune complexes were harvested with protein A, and immunoprecipitated proteins were analyzed by SDS-PAGE as above. Immunodetection was carried out using monoclonal anti-p21\(^{WAF-1}\) Ab-4 (1 μg/ml), monoclonal anti-PCNA (2 μg/ml), polyclonal anti-CDK2 (1 μg/ml), or polyclonal anti-CDK4 (1 μg/ml).

**Two-dimensional Gel Electrophoresis**—IgPhor two-dimensional gel equipment and reagents were obtained from Amersham Pharmacia Biotech. Ten million cells were lysed directly into rehydration/lysis buffer containing 8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 65 mM dithiothreitol, and 0.5% (v/v) immobilized pH gradient ampholites. For the first dimension, lysates were subjected to isoelectric focusing for 90 kVh on a precast pH gradient gel (pH 3–10 linear gradient). Second dimensional electrophoresis was carried out by standard SDS-PAGE on 15% gels, and p21\(^{WAF-1}\) was detected by Western blotting using polyclonal anti-p21\(^{WAF-1}\) (1 μg/ml) as described above.

**RESULTS**

**Cell Cycle Arrest Follows IL-3 Withdrawal in Cells with Activated v-Abl PTK**—Fig. 1 confirms our previous studies that in the presence of active v-Abl PTK (at 32 °C) there was no change in viable IC.DP cell number following the withdrawal of IL-3 (17). In contrast, in IC.DP cells with inactive v-Abl PTK (at 39 °C) and parental IC2.9 cells (with no v-Abl PTK), there was a decrease in viable cell number with time (Fig. 1 and Ref. 1). Analysis of cell cycle phase distribution in IC.DP cells after IL-3 withdrawal with v-Abl PTK active demonstrated that by 24 h there was a 10% increase of cells in G1 phase and a corresponding decrease in cells in G2 phase of the cell cycle, indicating that v-Abl allows the completion of ongoing rounds of DNA synthesis but no reinitiation from G1 (Table 1).

v-Abl PTK Up-regulates p21\(^{WAF-1}\) Protein Level—v-Abl PTK activation in the absence of IL-3 resulted in the accumulation of cells at the G1/S checkpoint. So we examined the effects of IL-3 withdrawal and/or v-Abl PTK activation on the cellular levels of the CDKs p21\(^{WAF-1}\). The DNA sequence of p21\(^{WAF-1}\) in IC.DP cells was confirmed as being wild type (data not shown). Fig. 2A shows that v-Abl PTK activation results in the up-regulation of p21\(^{WAF-1}\) as early as 3 h after IL-3 withdrawal, with a 5-fold increase observed by 24 h (mean, n = 3). Conversely, when v-Abl PTK was inactivated in IL-3-deprived IC.DP cells and parental IC.9 cells (no v-Abl PTK) at both 32 °C and 39 °C, there was no up-regulation of p21\(^{WAF-1}\); instead its level decreased as cells died. The readdition of IL-3 to IC2.9 cells that had been deprived of this cytokine for 2 h did not increase p21\(^{WAF-1}\) levels above basal levels (data not shown). Taken together these data show that the removal of the mitogenic stimulus of IL-3 per se did not cause a 5-fold increase in p21\(^{WAF-1}\), but that this resulted from v-Abl PTK signaling.

**IL-3 Drives Re-entry into Cell Cycle Despite Maintained High Levels of p21\(^{WAF-1}\)**—The most notable effect of v-Abl PTK shown in Fig. 2 was the suprastimulation of p21\(^{WAF-1}\) levels. Does this mean that the readdition of IL-3 would fail to stimulate stationary cells with v-Abl PTK active to re-enter the cell cycle? Would IL-3 decrease p21\(^{WAF-1}\) levels returning them to basal levels? To investigate this, cell population growth was monitored by measurement of viable cell number (Fig. 3A) and by S phase traverse following pulse labeling with BrdU (Fig. 3B). Viable cell number doubled 48 h after the readdition of IL-3 to cells with v-Abl PTK activity maintained. Immediately prior to IL-3 withdrawal there were 12% (12 ± 3%, n = 3) cells in S phase, 48 h after IL-3 withdrawal the percentage S phase cells was reduced to 6% (6 ± 2%, n = 3), but 24 h after the readdition of IL-3 to this stationary cell population, the percentage of cells moving through S phase was increased to 17% (17 ± 4%, n = 3). Fig. 3C demonstrates that despite this increase in cell cycle traverse, the v-Abl PTK-mediated up-regulation of p21\(^{WAF-1}\) was maintained. When v-Abl PTK was activated in cells continually cultured in the presence of IL-3, p21\(^{WAF-1}\) levels were elevated 6-fold (mean n = 3) despite continued cell proliferation (Fig. 3E). Parallel experiments with parental 2.9 cells (no v-Abl PTK) demonstrated that there were no temperature-dependent changes in the levels of p21\(^{WAF-1}\) (data not shown).

The levels and activities of CDK2 (which acts at both G/S and G2/M boundaries) and CDK4 (which acts only at G1/S) were assessed for cells deprived of IL-3 for 48 h and protected by v-Abl PTK and compared with those from cells that were re-stimulated with IL-3 for 48 h that had re-entered the cell cycle. CDK2 or CDK4 were immunoprecipitated, and the levels co-immunoprecipitated p21\(^{WAF-1}\) were analyzed by Western blotting. The levels of p21\(^{WAF-1}\)-associated with CDK2 and CDK4 when v-Abl PTK was activated was regardless of the presence or absence of IL-3 (Fig. 3D). Although p21\(^{WAF-1}\) levels were elevated in the protein complex, the protein expression levels of CDK2 and CDK4 were the same with or without IL-3 (Fig. 3D). The activity of CDK2 or CDK4 in these immunoprecipitates was assessed in vitro by measurement of substrate phosphorylation using histone H1. Predictably, CDK activities were high in the presence of IL-3 and lowered when the cytokine was not present.

**Fig. 4. Differential regulation of p21\(^{WAF-1}\) in cell cycle arrested and proliferating IC.DP cells containing activated v-Abl PTK**—\(A\), RT-PCR analysis of p21\(^{WAF-1}\) mRNA levels after v-Abl PTK activation in the presence of absence of IL-3. mRNA levels of p21\(^{WAF-1}\) in IC.DP with v-Abl PTK activated and incubated at the times shown in the presence of absence of IL-3 or in the presence of IL-3 after exposure to the topoisomerase II inhibitor etoposide (lane E). RT-PCR was performed using primers specific for β-actin and p21\(^{WAF-1}\) (see methods). Data shown are representative of four independent repeat experiments. B, effects of inhibition of transcription and translation on p21\(^{WAF-1}\) protein levels. IC.DP cells with active v-Abl PTK were treated either with actinomycin D or with cycloheximide (see “Experimental Procedures”) in the absence (panel i) or presence (panel ii) of IL-3. Western blot analysis p21\(^{WAF-1}\) protein expression was performed using cell lysates prepared after cell culturing for the times shown. The data shown are representative of four independent repeat experiments.
absent (Fig. 3D). These data prove that increased levels of p21WAF-1 bound to CDK2 or CDK4 do not always inhibit their activity. This finding necessarily implicates an IL-3-sensitive mechanism for inactivation of the activity of p21 WAF-1 as a CDKI.

p21WAF-1 Is Differentially Regulated in Nonproliferating and Proliferating IC.DP Cells with Active v-Abl PTK—To probe the mechanism of up-regulation of p21WAF-1 by v-Abl PTK and the effects of IL-3 upon this, we examined p21 WAF-1 mRNA levels using RT-PCR. An increase in p21 WAF-1 transcript was observed in IC.DP cells with v-Abl PTK active in nonproliferating cells in the absence of IL-3, when high levels of p21 WAF-1 protein were detected (Fig. 2A). However, in proliferating IC.DP cells in the presence of IL-3 with v-Abl PTK active and up-regulated p21 WAF-1 protein levels (Fig. 3E), there was no increase in p21 WAF-1 transcript (Fig. 4A). Treatment of IL-3-stimulated IC.DP cells with the topoisomerase II inhibitor etoposide resulted in the stabilization of p53 and up-regulation of p21 WAF-1 in the presence or absence of v-Abl PTK activity (data not shown). Here the elevation of p21 WAF-1 protein is mediated by transcription after DNA damage, but in this case, p21 WAF-1 transcription is not prevented by the IL-3 signal (Fig. 4A, lane E).

When transcription was inhibited with actinomycin D, the up-regulation of p21 WAF-1 protein was abolished in cells with v-Abl PTK active in the absence of IL-3 but not in the presence of IL-3 at 3 h (Fig. 4B, panel i). When translation was inhibited with cycloheximide, the up-regulation of p21 WAF-1 protein was abolished in cells both in the presence and absence of IL-3 (Fig. 4B, panels i and ii). Taken together these data implicate that both transcriptional and translational mechanisms drive the up-regulation of p21 WAF-1 by active v-Abl PTK in IL-3-deprived cells. However, inhibition of transcription by actinomycin D had no effect on the initial up-regulation of p21 WAF-1 by v-Abl PTK in the presence of IL-3. Thus, the increase in p21 WAF-1 protein levels at 3 h must be mediated via continued translation of existing p21 WAF-1 transcript. These data illustrate two distinct mechanisms that both lead to the elevation of p21 WAF-1 protein.
mediated up-regulation of p21WAF-1. However, in the presence of IL-3, no difference in p21WAF-1 was detectable, suggesting that the newly transcribed and newly synthesized p21WAF-1 protein had been modified to mask this C-terminal epitope. However, when IL-3 was present, a significant increase in p21WAF-1 associated immunoreactivity was detected using WA-1 in IC.DP cells, only WA-1 revealed changes in p21WAF-1 immunoreactivity after v-Abl PTK activation, and this was dependent on the presence of IL-3. Fig. 5B (panel i) illustrates that the increase in p21WAF-1 protein observed by Western blot was not detected using the C-terminal specific antibody WA-1 and flow cytometry in cell cycle-arrested IC.DP cells. The basal level of p21WAF-1 was still detectable, suggesting that the newly transcribed and newly synthesized p21WAF-1 protein had been modified to mask this C-terminal epitope. However, when IL-3 was present, a significant increase in p21WAF-1 associated immunofluorescence was detected using WA-1 (p < 0.01) in agreement with Western blot results (Figs. 5E and 5B, panel ii); in this cellular context the C terminus of p21WAF-1 was exposed on the newly synthesized protein. When v-Abl PTK was inactive, both in the presence and absence of IL-3 (Fig. 5B, panels iii and iv), no increase in p21WAF-1-associated fluorescence was detected, and a subpopulation of cells were observed in both cases that had no detectable p21WAF-1-associated fluorescence.

In addition, confocal microscopy was employed to confirm the IL-3-dependent changes in the availability for antibody binding to the p21WAF-1 C terminus observed by flow cytometry. Fig. 5C (panel i) demonstrates that in the absence of IL-3, the C terminus of p21WAF-1 was inaccessible to another C-terminal epitope-specific p21WAF-1 antibody SX118 despite v-Abl PTK-mediated up-regulation of p21WAF-1. However, in the presence of IL-3, the p21WAF-1 C-terminal epitope for SX118 binding is available (Fig. 5C, panel ii), and the up-regulated p21WAF-1 protein was detectable. Together these results indicate that in IC.DP cells with active v-Abl PTK, the C terminus of p21WAF-1 is occluded under conditions of cell cycle arrest and exposed during cell proliferation. This may reflect a difference in p21WAF-1 protein conformation or a difference in p21WAF-1 protein-protein interactions in the presence of IL-3 to reveal the C terminus.

To investigate the existence of different forms of p21WAF-1 in cell cycle arrested and proliferating IC.DP cells with active v-Abl PTK, cell lysates were subjected to two-dimensional gel electrophoresis. Fig. 5D illustrates that multiple forms of p21WAF-1 do indeed exist in IC.DP cells. Additionally, a different profile of isoforms of p21WAF-1 was observed when comparing lysates from cells stimulated by IL-3 to proliferate with those deprived of IL-3 and cell cycle arrested. Specifically, the presence of IL-3 consistently resulted in the loss of a basic isoform, spot 6, and the appearance of an additional acidic isoform, spot 1, and a neutral isoform, spot 3 when compared with the isoform profile of IL-3-deprived cells.

There Are No Differences in Nonproliferating Compared with Proliferating IC.DP Cells with Respect to Subcellular Localization of p21WAF-1 or Its Binding to CDKs or PCNA—p21WAF-1 has a putative nuclear localization signal at its C terminus and has been reported typically to be a nuclear protein (22). However, there are also reports that p21WAF-1 can be detected as a cytosolic protein (23). One explanation for the inactivity of high levels of p21WAF-1 in IL-3-stimulated IC.DP cells with v-Abl PTK active could be that it is sequestered in the cytosol. The subcellular localization of p21WAF-1 was examined in conditions of cell cycle arrest and proliferation in IC.DP cells with active v-Abl PTK using a polyclonal antibody to ensure the detection of all isoforms. The Ab-5 polyclonal anti-p21WAF-1 antibody was used to immunoprecipitate (IP) p21WAF-1. Data are representative of three independent repeat experiments.

The binding of p21WAF-1 to CDKs and to PCNA is important for p21WAF-1 mediated inhibition of cell cycle progression (11, 14). Therefore, we investigated the binding of p21WAF-1 to PCNA, CDK2, and CDK4 by coimmunoprecipitation from IC.DP cells with v-Abl PTK active. Fig. 7 illustrates that both in the presence and absence of IL-3, no differences in p21WAF-1 binding of PCNA, CDK2, and CDK4 were detected. These results were also confirmed by immunofluorescence and confocal microscopy (data not shown).

**DISCUSSION**

We set out to determine how cells deprived of IL-3 with v-Abl PTK activated accumulated in G1 phase of the cell cycle (Fig. 1 and Table I). We show that this cell cycle arrest is associated with v-Abl PTK-mediated up-regulation of the “universal"
which transcriptional activator(s) drive p21WAF-1 up-regulation
STAT3, and STAT5; reviewed in Ref. 24). We do not yet know
the progesterone receptor, E2F, and several STATs (STAT1,
the regulation of p21 WAF-1 by v-Abl
seem likely candidates.
are activated downstream of v-Abl PTK signaling (25), these
downstream of v-Abl PTK signaling; however, because STATs
in the presence of IL-3 it has a distinct function possibly as an
assembly factor and/or subcellular localization cue for CDK/
the presence or absence of IL-3 (Fig. 6).
We sought to determine at a molecular level how IL-3 sig-
naling bypasses the cell cycle arrest associated with the up-
regulation of p21 WAF-1 and inhibition of CDK2 and CDK4.

v-Abl PTK activation resulted in the transcriptional up-regu-
lation of p21 WAF-1 mRNA and protein level (Figs. 2A, 3E, and
4A). Initially p21 WAF-1 was identified as the main transcrip-
tional target of p53 (4). Protein stabilization of p53 follows DNA
damage in IC.DP cells (data not shown), but this did not occur
after IL-3 withdrawal with v-Abl active. Nevertheless, we can-
not rule out a p53-dependent mechanism for v-Abl PTK medi-
ated transcriptional up-regulation of p21 WAF-1. There are p53-
independent mechanisms to transcriptionally up-regulate p21 WAF-1
via a diverse group of transcription factors including the
progesterone receptor, E2F, and several STATs (STAT1,
STAT3, and STAT5; reviewed in Ref. 24). We do not yet know
which transcriptional activator(s) drive p21 WAF-1 up-regulation
downstream of v-Abl PTK signaling; however, because STATs
are activated downstream of v-Abl PTK signaling (25), these
seem likely candidates.

v-Abl PTK-mediated up-regulation of p21 WAF-1 protein level
was further increased by the readdition of IL-3, and this was
concomitant with entry to the cell cycle (Fig. 3, A and C).
In the presence of this mitogen, p21 WAF-1 up-regulation was not transcrip-
tional (Fig. 4, A and B, panel ii). Several transcription
independent mechanisms to up-regulate p21 WAF-1 have been
reported. Increased stabilization of p21 WAF-1 mRNA has led to
up-regulated p21 WAF-1 protein, for example, by binding of the
Elav-like protein HuD to the transcript (26). Increased protein
stability contributed to increased levels of p21 WAF-1 by direct
inhibition of the proteosome (27) or binding and abrogation of
PCNA function, thus preventing progression through the
proteosome (28). However, neither of these mechanism(s) are con-
sistent with our observations in IL-3 replete IC.DP cells with v-
Abl PTK activated, implying another route to p21 WAF-1, pos-
sibly by increase translation of existing mRNA, although fur-
ther work is required to confirm this. IL-3 does not suppress
the transcription of p21 WAF-1 in every cellular context as shown
in Fig. 4A (lane E) where the etoposide damage signal main-
tains p21 WAF-1 transcription in the presence of IL-3.

One of the striking questions posed by our studies is how
IC.DP cells proliferate at the same rate with either basal levels of
p21 WAF-1 (with IL-3 but inactive v-Abl PTK) or with 6-fold
up-regulated levels of p21 WAF-1 (with IL-3 and active v-Abl
PTK), where in the latter case p21 WAF-1, although associated in
increased amount with CDK4 and CDK2 (Fig. 3D), does not function as a CDKI. We showed that the p21 WAF-1 protein
up-regulated in proliferating IC.DP cells exists in a different
conformation to that in cell cycle arrested cells, reflected by
different C-terminal epitope availability (Fig. 5, B and C). This
suggests either different conformational forms of p21 WAF-1
and/or a change in protein-protein interactions. Our studies
using two-dimensional gel electrophoresis demonstrate that
there are several isoforms of p21 WAF-1 and that the isoform
profile is different in IC.DP cells with v-Abl PTK active in the
presence or absence of IL-3 (Fig. 5D). We are currently purs-
uing the identity of these isoforms. Post-translational modifi-
cation of p21 WAF-1 at its C terminus can occur; phosphorylation in
the PCNA binding domain of p21 WAF-1 has been recently re-
ported (29), and post-translational modification might affect its
subcellular location and/or the binding of p21 WAF-1 to other
proteins. However, we have not detected any difference in the
subcellular location or the binding of several established bind-
ing partners of p21 WAF-1 (Fig. 6, 7), and these parameters could
not therefore account for the changes in epitope availability
monitored by flow cytometry and confocal microscopy (Fig. 5, B
and C). Other p21 WAF-1 binding proteins have been identified
including GADD45 (30), stress-activated protein kinases (31),
and apoptosis signaling kinase 1 (23), but the effects of these
binding partners on p21 WAF-1 function as a CDKI are currently
unknown. Interestingly, the protein phosphatase inhibitor SET
binds the C terminus of p21 WAF-1 (in the region of the WA1 and
SX118 binding sites), abrogating its function as an inhibitor of
CDK2 specifically bound to cyclin E (32). We were unable to
demonstrate SET binding to p21 WAF-1 in IC.DP cells cultured
with or without IL-3 (data not shown).
In summary, we propose a model whereby v-Abl PTK rein-
forces cell cycle arrest in the absence of IL-3 that is associated
with the transcriptional up-regulation of p21 WAF-1 protein, in a

FIG. 8. Schematic diagram showing the regulation of p21 WAF-1 by v-Abl
PTK and IL-3 during the decision fork between cell cycle arrest and
CDKI, p21 WAF-1 (Fig. 2A). After IL-3 withdrawal in the absence
of v-Abl PTK activity, there was no up-regulation of p21 WAF-1
at any time before the onset of cell death (Fig. 2). Thus, the
activation of v-Abl PTK rather than the withdrawal of the mitogen per se caused the observed increase in this CDKI.
p21 WAF-1 has been shown to have a dual role in the regu-
lation of CDK4 kinase activity: Transfection of p21 WAF-1 into
U2OS cells resulted in the assembly of kinase active CDK4/
cyclin D1 complexes (7). Here we show that in the presence of
v-Abl PTK and IL-3 cells proliferate but the up-regulated
p21 WAF-1 fails to inactivate the kinase activity of both CDK4
and CDK2 (both of which remain at basal expression levels).
p21 WAF-1 function may therefore vary depending on the incom-
ing signal(s): when it is up-regulated by v-Abl PTK signaling in
the absence of IL-3, p21 WAF-1 functions as a CDKI, whereas in
the presence of IL-3 it has a distinct function possibly as an
assembly factor and/or subcellular localization cue for CDK/
cyclins, although we found no evidence of any difference in the
subcellular distribution of p21 WAF-1 itself in the presence and
absence of IL-3 (Fig. 6).

Downloaded from http://www.jbc.org/ by guest on July 23, 2018
form bound to PCNA and CDK2 or CDK4 that functions as a CDKI (Fig. 8). In conditions of IL-3-driven cell proliferation, the transcriptional up-regulation of p21WAF-1 by v-Ab1 PTK is abrogated, yet p21WAF-1 protein is maintained at elevated levels. However, these high levels of p21WAF-1 molecules in proliferating cells exist in a different form that is functionally inactive with respect to CDK inhibition. We are currently investigating the mechanism of inactivation of p21WAF-1 by IL-3 with respect to CDKI function in proliferating cells expressing elevated levels of this cell cycle regulator.

Although it may seem counter-intuitive that an oncogenic tyrosine kinase reinforces cell cycle arrest, we speculate that there are circumstances in which this might be advantageous for a tumor cell (e.g. when it is located in a hostile microenvironment with limiting growth/survival factors). Entry to cell cycle in the absence of appropriate mitogenic stimuli promotes apoptosis (33). Therefore, the rapid imposition of p21WAF-1 driven G1 arrest may be important in the mechanism whereby v-Ab1 PTK maintains cell viability during the period prior to the up-regulation of the anti-apoptotic protein Bcl-xL (17). When p21WAF-1 up-regulation was abrogated in HCT116 colon carcinoma cells using an antisense approach, instead of growth arrest after irradiation cells committed to apoptosis (34). If we can understand how growth arrest associated with up-regulated levels of p21WAF-1 is overridden by incoming signals from mitogens, this information might be exploitable therapeutically to modulate the cellular decision between growth arrest and apoptosis. In situations where tumor cells survive after drug-induced damage because they can undergo p21WAF-1-mediated cell cycle arrest and buy time to repair the damage, they may instead be pushed through the cell cycle via inactivation of p21WAF-1 function and commit to apoptosis as they attempt replication on a damaged DNA template.

Acknowledgments—We thank Boris Vojtesek for the gift of the WA-1 antibody and Duncan Smith for guidance with two-dimensional gels. We thank John Hickman, Andy Koff, Nic Jones, Guy Makin, and Bernard Corfe for constructive criticism of this manuscript.

REFERENCES

1. Evans, C. A., Owen-Lynch, P. J., Whetton, A. D., and Dive, C. (1993) Cancer Res. 53, 1735–1738
2. Morgan, D. O. (1995) Nature 374, 131–134
3. Xiong, Y., Zhang, H., and Beach, D. (1992) Cell 71, 505–514
4. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) Cell 75, 817–825
5. Harper, J. W., Adam, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) Cell 75, 805–816
6. Xiong, Y., Hannon, G. J., Zhang, H., Caso, D., Kobayashi, R., and Beach, D. (1993) Nature 366, 701–704
7. LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Pattea, A., and Harlow, E. (1997) Genes Dev. 11, 847–862
8. Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M., and Sherr, C. J. (1999) EMBO J. 18, 1571–1583
9. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leeder, P. (1995) Cell 82, 675–684
10. Dulic, V., Stein, G. H., Far, D. F., and Reed, S. I. (1998) Mol. Cell. Biol. 18, 546–557
11. Cayrol, C., Knuebokler, M., and Ducommun, B. (1998) Oncogene 16, 311–320
12. Waldman, T., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1996) Nature 381, 713–716
13. Deleted in proof
14. Ogryzko, V. Y., Wong, P., and Howard, B. H. (1997) Mol. Cell. Biol. 17, 4877–4882
15. Medema, R. H., Klompman, R., Smits, V. A. J., and Rijksen, G. (1998) Oncogene 16, 431–441
16. Nakanishi, N., Adami, G. R., Robetorye, R. S., Noda, A., Venable, S. F., Dimitrov, D., Perererra-Smith, O. M., and Smith, J. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4352–4356
17. Chen, Q., Turner, J., Watson, A. J. M., and Dive, C. (1997) Oncogene 15, 2249–2254
18. Kipros, L. E., Lee, G. J., and Wang, J. Y. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1345–1349
19. Karasuyama, H., and Melchers, P. (1988) Eur. J. Immunol. 18, 97–104
20. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 481–501, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Dulic, V., Lees, E., and Reed, S. I. (1992) Science 257, 1958–1961
22. Duttaroy, A., Qian, J. F., Smith, J. S., and Wang, E. (1997) J. Cell. Biochem. 66, 434–446
23. Asada, A., Yamada, T., Ichii, H., Delia, D., Miyazono, K., Fukumuro, K., and Mizutani, S. (1999) EMBO J. 18, 1223–1234
24. Garret, A. L., and Tyner, A. L. (1999) Exp. Cell. Res. 246, 280–289
25. Daniel, N. N., Pernis, A., and Rothman, P. B. (1995) Science 269, 1875–1877
26. Joseph, B., Orlian, M., and Furneaux, H. (1998) J. Biol. Chem. 273, 20511–20516
27. Blagosklonov, M. V., Wu, G. S., Osuara, S., and El-Deiry, W. S. (1996) Biochem. Biophys. Res. Commun. 227, 564–569
28. Cayrol, C., and Ducommun, B. (1998) Oncogene 17, 2457–2444
29. Scott, M. T., Morsie, N., and Ball, K. L. (2000) J. Biol. Chem. 275, 11529–11537
30. Kearsley, J. M., Coates, P. J., Precott, A. R., Warbrick, E., and Hall, P. A. (1995) Oncogene 11, 1675–1683
31. Shima, J., Lee, H., Park, J., Kim, H., and Choi, E.-J. (1996) J. Biol. Chem. 271, 804–807
32. Estanyol, J. M., Jaumot, M., Casanovas, O., Rodriguez-Villarrupa, A., Agell, N., and Bache, O. (1999) J. Biol. Chem. 274, 33161–33165
33. Colombel, M., Olsson, C. A., Ng, P. Y., and Buttyan, R. (1992) Cancer Res. 52, 4313–4319
34. Tian, H., Wittmack, E. K., and Jorgensen, T. J. (2000) Cancer Res. 60, 679–684
v-Abl Protein-tyrosine Kinase Up-regulates p21WAF-1 in Cell Cycle Arrested and Proliferating Myeloid Cells
Sophia J. Khanna, Robin Brown, Anthony D. Whetton, Kathryn L. Ball and Caroline Dive

J. Biol. Chem. 2001, 276:11143-11150.
doi: 10.1074/jbc.M007073200 originally published online November 29, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007073200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 13 of which can be accessed free at http://www.jbc.org/content/276/14/11143.full.html#ref-list-1