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Characterization of Drug Resistance Mediated via the Suppression of Apoptosis by Abelson Protein Tyrosine Kinase

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SUMMARY

Constitutive activation of the Abelson (Abl) protein tyrosine kinase (PTK) is a causative event in chronic myeloid leukemia, where intense chemotherapy currently fails to eradicate the leukemic clone. Using a mouse mast cell line (IC.DP), we previously showed that v-Abl PTK induced resistance to the anticancer drugs melphalan and hydroxyurea by the suppression of apoptosis. Here, using this cell line, we demonstrate by alkaline elution that v-Abl PTK did not affect the levels of DNA damage induced by either drug. This confirms that v-Abl PTK acts downstream of the drug-target interaction to prevent the coupling of drug-induced damage to the apoptotic pathway. Although Abl PTK- and interleukin-3 (IL-3)-stimulated signaling events share common signaling pathways, a similar level of drug resistance was not provided by IL-3, implying that Abl PTK does not merely mimic an IL-3 survival signaling pathway. Previously we demonstrated translocation of protein kinase C-β_{II} stimulated by activation of Abl PTK. Drug sensitivity was restored in cells with active v-Abl PTK by simultaneous addition of calphostin C, an inhibitor of protein kinase C, suggesting a role for protein kinase C in the suppression of drug-induced apoptosis by v-Abl PTK. One novel strategy for the treatment of chronic myeloid leukemia could therefore include the use of a downstream modifier of the Abl PTK-mediated survival signaling pathway to render leukemic cells more sensitive to a second drug, such as a cytotoxic agent.

The Abl murine leukemia virus can acutely transform hemopoietic cells *in vivo* (1). *In vitro* it promotes survival and/or proliferation of growth factor-dependent hemopoietic cell lines in the absence of the required growth factor (2). The transforming activity of the virus is dependent on the PTK activity of the v-*abl* oncogene (3). Similarly, the *bcr-abl* oncogene-mediated transformation of hemopoietic cells is dependent upon the PTK activity of the Abl moiety of the chimeric protein (4). The *bcr-abl* oncogene is characteristically expressed in CML as a consequence of a chromosomal translocation leading to formation of the Ph chromosome (reviewed in Ref. 5). When expressed in multipotent hemopoietic cells *in vivo*, *bcr-abl* and v-*abl* have similar effects (1). Although overt transformation of hemopoietic progenitor cells to growth factor-independent proliferation does not appear to occur in CML, there is some evidence for increased cell survival in the absence of cytokines (6), as well as drug resistance (7). During the chronic phase of CML there is increasing resistance to hydroxyurea and busulphan (7). These drugs have been used for the palliative treatment of CML; they relieve the granulocytosis observed (presumably by partially inhibiting cell divisions downstream from the Ph^+ clonal stem cell). However, in the past more intensive chemotherapy has failed to remove the Ph^+ stem cell population, implying that the most important Ph^+ cells, the stem cells, display some form of drug resistance. Many mechanisms of anticancer drug resistance, at the level of the drug-target interaction, have already been described (8), but the drug-target interaction is not the sole determinant of the response of cells to a drug (9). Cells treated with anticancer agents with disparate modes of action die by the conserved process of apoptosis or programmed cell death (10). The suppression of the apoptotic pathway downstream of the drug-target interaction could therefore be an important novel mechanism of drug resistance (11, 12). Even a slightly increased resistance to chemotherapeutic agents could be of major significance *in vivo*, and thus we have investigated the molecular mechanisms underlying this phenomenon in Abl PTK-expressing cells.

We have previously shown, using an IL-3-dependent mouse mast cell line (IC2.9) transfected with a temperature-sensitive form of the v-Abl PTK (IC.DP subclone), that v-Abl PTK can induce cell survival without proliferation after with-

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ABBREVIATIONS: Abl, Abelson; PTK, protein tyrosine kinase; CML, chronic myeloid leukemia; Ph, Philadelphia; TdT, terminal deoxynucleotidyl transferase; FITC, fluorescein isothiocyanate; PKC, protein kinase C; PBS, phosphate-buffered saline; IL-3, interleukin-3.

drawal of IL-3 (13). Furthermore, we demonstrated that treatment of cells with melphalan or hydroxyurea resulted in death by apoptosis when v-Abl PTK was inactive but activation of v-Abl PTK by temperature switching suppressed drug-induced apoptosis, rendering the cells drug resistant (14) and therefore similar to CML cells during the accelerated phase of the disease leading up to blast crisis. Similar results have been obtained using v-*abl* and *bcr-abl* in other model systems (15), and resistance to drug-induced apoptosis has also been demonstrated in cells from patients with CML (16). A sensor of DNA damage that is associated with the induction of apoptosis is the tumor suppressor gene *p53* (17). One recent report that implies that Abl PTK might suppress drug-induced apoptosis downstream of the drug-target interaction describes the finding that Bcr-Abl PTK activity alters the conformation of wild-type p53 from the suppressor form to the promoter form (18), i.e., it may affect drug-induced apoptosis at the level of the sensor of DNA damage.

In this study we have investigated the mechanism of suppression of drug-induced apoptosis by v-Abl PTK and compared the effects of Abl PTK with those of IL-3. We demonstrate a possible mechanism for Abl PTK-mediated suppression of drug-induced apoptosis.

Materials and Methods

Reagents. Unless otherwise stated, all reagents were obtained from Sigma (Poole, UK). Recombinant IL-3 was from R&D Systems (Oxford, UK).

Cell culture. IC2.9 and IC.DP cells were cultured in Fischer's medium supplemented with 5% X63-Ag-653 cell-conditioned medium containing IL-3 (19), 10% (v/v) horse serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. Cultures were maintained at 37° with 5% CO_2.

Assessment of apoptosis using the TdT assay and multiparameter flow cytometry. Cell samples were prepared as described Gorczyca *et al.* (20), by fixation first in formaldehyde (1%, v/v) and then in ethanol (70%, v/v). Cells were rehydrated in PBS, resuspended in a cacodylate buffer containing 5 units of TdT and 0.5 nM biotinylated dUTP (Boehringer Mannheim Biochemicals, Indianapolis, IN), and incubated at 37° for 30 min. Cells were rinsed in PBS, resuspended in 100 μl of saline sodium citrate buffer containing FITC-conjugated avidin (5 μg/ml), Triton X-100 (0.1%, v/v), and nonfat dried milk (5% w/v), and incubated at room temperature in the dark for 30 min. Cells were rinsed in PBS containing 0.1% (v/v) Triton X-100 and resuspended in PBS before addition of propidium iodide (32 μM) 1 min before analysis by flow cytometry. A Becton Dickinson FACSVantage flow cytometer (Becton Dickinson, San Jose, CA) equipped with an Enterprise laser (Innova Technology, Coherent Inc., Palo Alto, CA) was set to excite at 150 mW, using the 488-nm line. For each sample, 10^4 cells were analyzed. Forward and orthogonal light scatter was collected together with green fluorescence (at 530 ± 30 nm; TdT-incorporated FITC-avidin-biotinylated dUTP) and red fluorescence (575 ± 22 nm; propidium-DNA). Data were acquired and analyzed using Lysis II software (Becton Dickinson). All experiments were performed three times.

Assessment of drug-induced DNA damage. Melphalan-induced DNA cross-links and hydroxyurea-induced single-strand breaks were measured by alkaline elution as described previously (21). Cells were labeled for 24 hr with [^{14}C]thymidine (specific activity, 52 mCi/mmol; Amersham International) at 39° (to ensure inactivation of v-Abl PTK in IC.DP cells) (22), washed, and incubated for 2 hr with isotope-free medium at 32° (to activate v-Abl PTK) (22) or at 39°. Cells were then exposed to hydroxyurea (1 mM, for 24 hr) or melphalan (10 μM, for 18 hr). Before elution, melphalan-treated cells were exposed to 300 rad of X-irradiation at 0°, as a means of introducing a controlled number of single-strand breaks. Cells (8×10^5) were collected on a polycarbonate filter (pore size, 2 μm; diameter, 25 mm; Whatman International). Cells were lysed on the filter with 5 ml of lysis solution (0.2% sodium lauryl sarkosine, 2 M NaCl, 0.04 M EDTA, pH 10), followed by proteolytic digestion with proteinase K (0.5 mg/ml, in lysis solution). The filters were washed with 0.02 M EDTA, pH 10. Elution was carried out in the dark, using a solution of 0.02 M EDTA adjusted to pH 12.1 with tetraethylammonium hydroxide (35% solution in water; Aldrich), at a flow rate of 0.04 ml/min. Fractions were collected, for scintillation counting, at 3-hr intervals for at least 15 hr. The level of cross-linking induced by melphalan was quantitated in terms of the cross-linking coefficient (K_c), defined as $K_c = [(1 - r_o)/(1 - r)]^{1/4} - 1$, where r and r_o are the fractions of the DNA retained on the filters for drug-treated and untreated cells, respectively (23). The levels of single-strand breaks produced by hydroxyurea were standardized by the calculation of an equivalent dose of X-irradiation (rad equivalents) (21).

Isolation of cell cycle fractions by counterflow centrifugal elutriation. IC.DP cells from an exponential growth phase in culture were maintained at 39° for 18 hr to ensure inactivation of v-Abl PTK (22). Elutriation was carried out at 4° in Fischer's medium containing 5% (v/v) horse serum, as described previously (24). Cells from either G_1 or $S/G_2/M$ fractions were resuspended in Fischer's medium containing IL-3 and serum (as described above), treated with melphalan (10 μM), and maintained at 39° or switched from 39° to 32° at the times indicated. Cell death was assessed by measuring the membrane permeability of cells to trypan blue (0.2%, w/v); the mode of cell death was apoptosis, as was previously characterized in detail (14). To assess the cell cycle distribution of each fraction, 5×10^5 cells were fixed in ethanol (70%, v/v, in PBS) and stained with propidium iodide (32 μM) for 1 min. The resulting DNA-propidium red fluorescence (of 10^4 cells) was analyzed using a Becton Dickinson FACSVantage flow cytometer. Results were analyzed using Becton Dickinson Cell Fit software.

Treatment of cells with calphostin C. Logarithmic phase cells were cultured at 2×10^5/ml at 32° for 2 hr to activate v-Abl PTK (22) and were treated with either melphalan (10 μM) or hydroxyurea (10 mM) and calphostin C (25–250 ng/ml). After exposure to hydroxyurea for approximately one cell cycle (24 hr), cells were resuspended in fresh medium (containing calphostin C but without hydroxyurea) at the original seeding density. Cell viability was determined by trypan blue (0.2%, w/v) exclusion.

Results

Suppression of drug-induced apoptosis by v-Abl PTK. We have previously shown that apoptosis induced by the anticancer drugs melphalan and hydroxyurea is suppressed in IC.DP cells expressing a temperature-sensitive v-Abl PTK (14). We confirmed that the mode of drug-induced cell death was apoptosis using the TdT assay and flow cytometry (Fig. 1). For the remainder of the experiments described below, we have used trypan blue exclusion as a measure of cell death, because we have previously shown that this correlates with the cumulative effects of apoptosis in these cell populations (13, 14).

v-Abl PTK does not modulate the levels of drug-induced DNA damage. The effects of v-Abl PTK activation on the acquisition of melphalan- or hydroxyurea-induced DNA damage were measured by alkaline elution (21). DNA interstrand cross-linking results in retardation of the elution rate of DNA after X-irradiation; this was apparent in melphalan-treated IC2.9 and IC.DP cells at either temperature, regardless of v-Abl PTK activation (Fig. 2A). The cross-linking coefficient (K_c) values derived from these experiments are

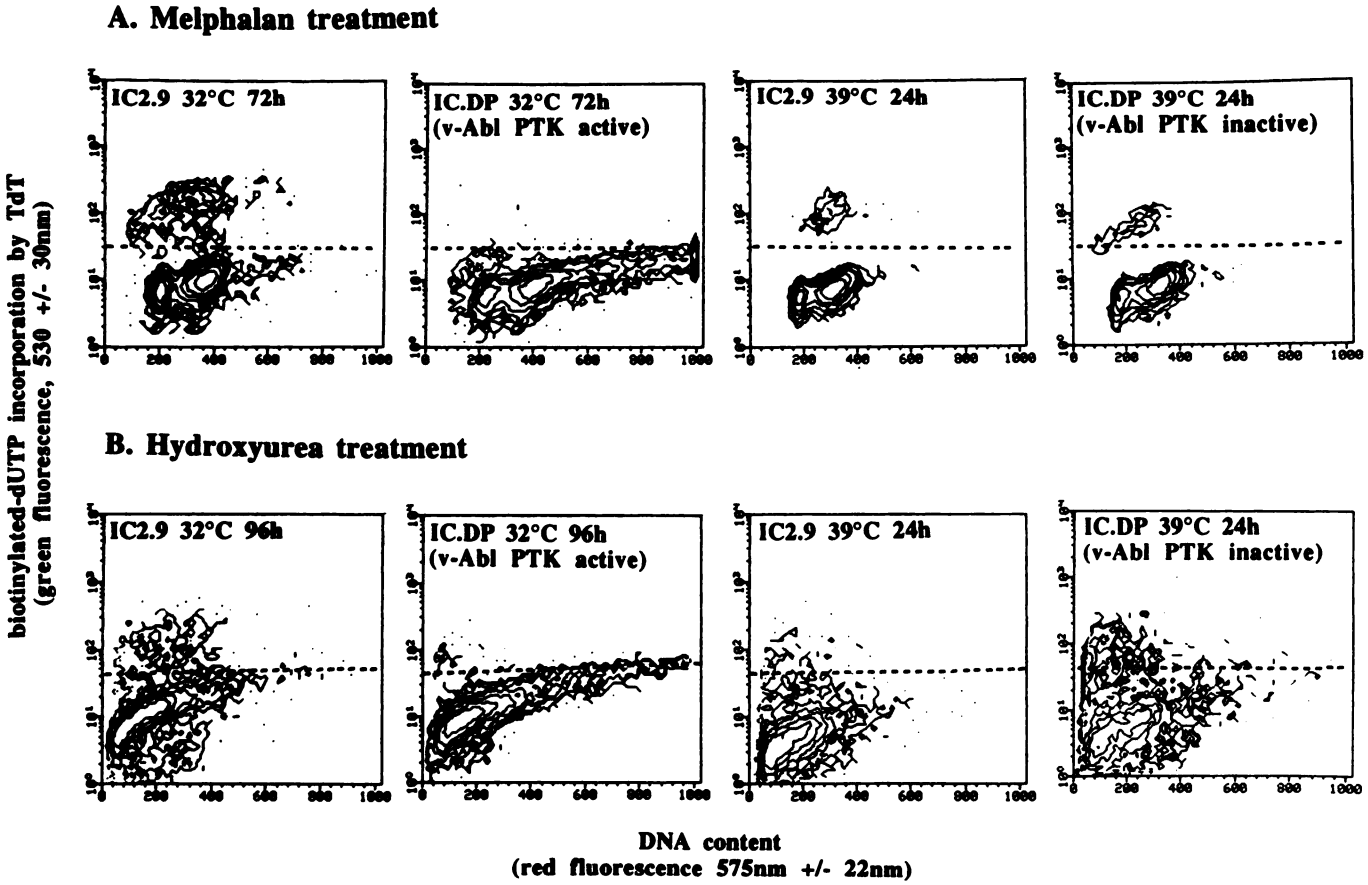

Fig. 1. Measurement of apoptosis using the TdT assay and flow cytometry. Incorporation of biotinylated dUTP, by exogenous TdT, into the DNA of cells exposed to the DNA-alkylating agent melphalan (A) or hydroxyurea (B) was determined. Two-dimensional frequency contour plots of red fluorescence (propidium-stained DNA) versus green fluorescence (TdT-incorporated FITC-avidin-biotinylated dUTP) are shown. Cells with green fluorescence *above the dotted line* were considered positive for the TdT assay. In each experiment <1% of untreated cells exhibited strong green fluorescence (data not shown). Results are for 10^4 cells and are representative of three repeated experiments. A, IC.DP cells were exposed to 10 μM melphalan for 24 or 72 hr at 39° (v-Abl PTK inactive) or 32° (v-Abl PTK active), respectively. B, IC.DP cells were exposed to 10 mM hydroxyurea for 24 hr, washed, and resuspended in drug-free medium, and flow cytometric analysis was performed at 24 hr for cells at 39° or at 96 hr for cells at 32°. In each experiment IC2.9 cells (no v-Abl PTK) were treated identically.

shown in Table 1. There was no significant difference in the levels of melphalan-induced cross-links between IC2.9 and IC.DP cells at either 32° or 39° (Student's t test, $p > 0.05$).

Hydroxyurea inhibits the enzyme ribonucleotide reductase, which is involved in the synthesis of bases for DNA, and thereby indirectly induces DNA single-strand breaks (25). The levels of DNA strand breaks induced by hydroxyurea were unaffected by v-Abl PTK activity (compare parental IC2.9 cells and IC.DP cells at 32° in Fig. 2B and Table 2). At 39° a significant difference between the IC2.9 and IC.DP cells was seen, despite the absence of v-Abl PTK activity in both cell types (22), but at this temperature there was actually more cell death in the IC.DP cells treated with hydroxyurea at the time of elution, compared with that observed in IC2.9 cells (data not shown). This cell death was evident as DNA fragmentation in the pre-elution fractions, as can be seen at 0 hr in Fig. 2B. There was also a significant difference in the frequency of single-strand breaks produced at 39°, compared with 32°, in both cell types. This is probably due to differences in ribonucleotide reductase activity and the cell cycle duration at the two different temperatures and is reflected by the rate of cell death after hydroxyurea treatment (14). Although fewer DNA single-strand breaks were observed in IC2.9 cells at 32°, compared with 39°, and the kinetics of cell death reflected this difference, IC2.9 cells at 32° still coupled this DNA damage to apoptosis (Fig. 1B), in contrast to cells protected by v-Abl PTK.

Suppression of apoptosis by v-Abl PTK is independent of cell cycle distribution. To investigate whether the suppression of apoptosis by v-Abl PTK is dependent on its activity in one particular phase of the cell cycle, IC.DP cells were separated by centrifugal elutriation into fractions enriched for either G_1-phase cells or a combination of S- and G_2/M-phase cells. Cells were treated with melphalan (10 μM) at time 0, and v-Abl PTK was activated at the specified times by a temperature switch from 39° to 32°. The resultant kinetics of cell death are shown in Fig. 3. The cell cycle profiles show the cell cycle distribution of melphalan-treated cells at the time of activation of v-Abl PTK (i.e., the temperature switch); cell cycle distribution statistics are shown in Table 3. Control cells in both fractions proliferated during the experiment (approximately 10-fold increase in 144 hr), but no proliferation was observed with melphalan-treated cells (data not shown). Cells at 39° (with v-Abl PTK inactive) treated with melphalan died over a 96-hr time course (Fig. 3, *1*), confirming our previously reported observations (14). However, our most important observation was that there was no significant difference in the kinetics of cell death seen with

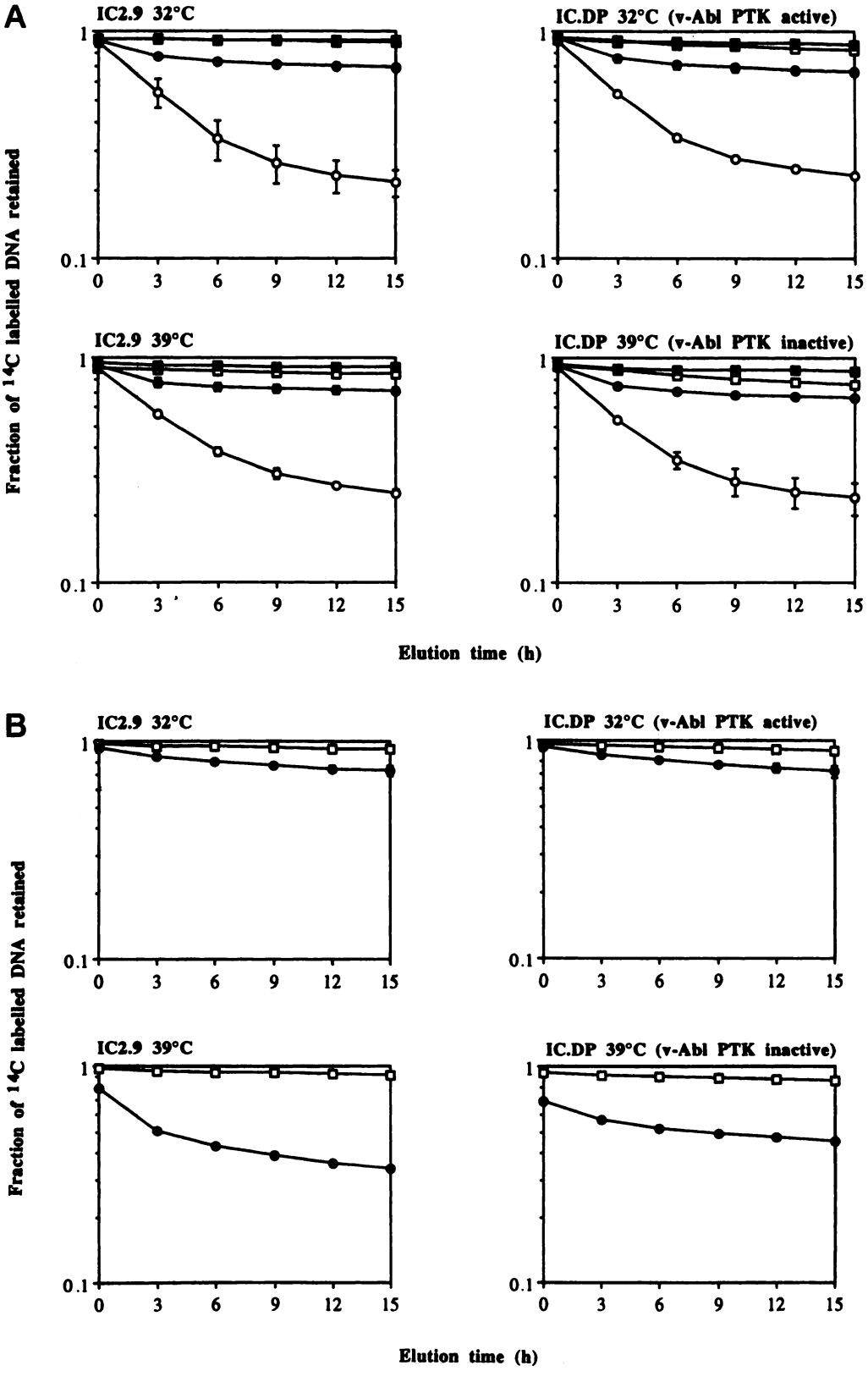

Fig. 2. A, Alkaline elution patterns of DNA from unirradiated and X-irradiated IC2.9 and IC.DP cells after treatment with melphalan (10 μM). Cells were treated for 18 hr before elution at 32° or 39° (permissive and restrictive temperatures, respectively, for v-Abl PTK activity in IC.DP cells). The graphs show the fraction of [^{14}C]thymidine retained on the filter; duplicate samples were performed for each experiment and the mean and high and low values are shown (*bars*). One representative experiment of three is shown. □, Control cells (no strand breaks); ○, X-irradiated control cells (known number of strand breaks); ■, melphalan-treated cells (no strand breaks); ●, X-irradiated melphalan-treated cells (strand breaks but elution slowed by cross-linking). B, Alkaline elution patterns of DNA from IC2.9 and IC.DP cells after treatment with hydroxyurea (1 mM). Cells were treated for 24 hr before elution at 32° or 39° (permissive and restrictive temperatures, respectively, for v-Abl PTK activity in IC.DP cells). The graphs show the fraction of [^{14}C]thymidine retained on the filter; duplicate samples were performed for each experiment and the mean and high and low values are shown (*bars*). One representative experiment of three is shown. □, Control cells (no strand breaks); ●, hydroxyurea-treated cells (drug-induced strand breaks).

the cells enriched for G_1 or for S/G_2M phases of the cell cycle (Student's *t* test, $p > 0.05$). Cells treated with melphalan at 32°, when v-Abl PTK was active, maintained their viability during the experiment, with <30% dead after 144 hr (Fig. 3, *2*). Again, there was no significant difference in the kinetics of cell death observed with the cells enriched for G_1 or for S/G_2M phases of the cell cycle (Student's *t* test, $p > 0.05$). The difference observed in cell death between IC.DP cells at 39° and those at 32° (Fig. 3, *1* and *2*) was not simply a reflection of temperature, because the percentage of IC2.9 cells at 32° that had died was >95% at 144 hr (14). This death of IC2.9 cells at 32° is also apparent in Fig. 1A.

TABLE 1

DNA damage in IC2.9 and IC.DP cells after treatment with melphalan

Elution values at 9 hr of DNA from unirradiated and X-irradiated IC2.9 and IC.DP cells after treatment with melphalan (10 μM). Cells were treated for 18 hr before elution at 32° or 39° (permissive and restrictive temperatures, respectively, for v-Abl PTK activity in IC.DP cells). Calculation of the cross-linking coefficient is described in Materials and Methods. The results shown are the average ± standard error of three repeated experiments. Significance between IC2.9 and IC.DP cells at the same temperature was calculated using the paired Student's *t* test.

Cell line/temperature	Elution value at 9 hr				Cross-linking coefficient (K_c)	
	Control		Melphalan-treated			
IC2.9/32°	0.913 ± 0.008	(p = 0.169)	0.919 ± 0.004	(p = 0.592)	121.8 ± 15.2	(p = 0.250)
IC.DP/32° (v-Abl PTK active)	0.878 ± 0.014		0.913 ± 0.014		95.8 ± 20.1	
IC2.9/39°	0.883 ± 0.013	(p = 0.090)	0.899 ± 0.013	(p = 0.075)	173.6 ± 19.0	(p = 0.153)
IC.DP/39° (v-Abl PTK inactive)	0.846 ± 0.022		0.879 ± 0.007		105.1 ± 12.0	

TABLE 2

DNA damage in IC2.9 and IC.DP cells after treatment with hydroxyurea

Elution values at 9 hr of DNA from IC2.9 and IC.DP cells after treatment with hydroxyurea (1 mM). Cells were treated for 24 hr before elution at 32° or 39° (permissive and restrictive temperatures, respectively, for v-Abl PTK activity in IC.DP cells). The results shown are the average ± standard error of three repeated experiments. Significance between IC2.9 and IC.DP cells at the same temperature was calculated using the paired Student's *t* test.

Cell line/temperature	Control elution value at 9 hr		Hydroxyurea-treated rad equivalents at 9 hr	
IC2.9/32°	0.914 ± 0.010	(p = 0.349)	36.0 ± 7.0	(p = 0.423)
IC.DP/32° (v-Abl PTK active)	0.900 ± 0.014		31.0 ± 2.1	
IC2.9/39°	0.914 ± 0.010	(p = 0.203)	157.0 ± 13.9	(p = 0.009)
IC.DP/39° (v-Abl PTK inactive)	0.890 ± 0.003		112.0 ± 9.5	

When cells were cultured at 39° (with v-Abl PTK inactive) for specified periods of time and then switched to 32° to activate v-Abl PTK (Fig. 3, *3–6*), there was a shift in cell cycle distribution of the G_1-enriched fraction to S/G_2M (apparent after 20 hr), whereas some of the S/G_2M-enriched population appeared to enter G_1 (an increase in G_1 could be seen at 5 hr). v-Abl PTK was activated at intervals after melphalan addition (2–20 hr). Despite the differences in cell cycle distribution, there were still no significant differences observed between the two enriched fractions in either the degree or kinetics of suppression of cell death by v-Abl PTK (Student's *t* test, $p > 0.05$).

v-Abl PTK-mediated drug resistance cannot be mimicked by IL-3. Activation of Abl PTK and activation of the IL-3 receptor PTK elicit some common signaling events in IC.DP cells (26). To determine whether the drug resistance we have reported is merely due to superstimulation of molecular mechanisms normally activated by IL-3 (and therefore of little consequence in disease progression induced by Abl PTK), we have cultured IC2.9 cells with high concentrations of IL-3. IC2.9 cells were treated with melphalan (1–10 μM) and 500 units/ml IL-3 (10 times the normal cell culture concentration). The level of cell death seen after treatment with melphalan was unaffected by increased concentration of IL-3 (Fig. 4). Moreover, there were no significant differences in either the concentration-dependent response to melphalan or the kinetics of cell death with 10 μM melphalan (Student's *t* test). Thus, the effects of Abl PTK we observed are not due to activation of molecular events in a fashion similar to that of an hemopoietic growth factor. The kinetics of cell growth were unaffected when the concentration of IL-3 was increased from 50 units/ml to 500 units/ml (data not shown).

Calphostin C attenuates v-Abl PTK-mediated drug resistance. We have previously demonstrated that the PKC inhibitor calphostin C can restore apoptosis to IL-3-deprived IC.DP cells while v-Abl PTK is active (27). Activation of v-Abl PTK resulted in the translocation of PKC-β_{II} to the nuclear periphery, and this translocation was also prevented by calphostin C (27). Calphostin C was used in preference to other inhibitors such as staurosporine because it is a relatively selective inhibitor of PKC, with a much reduced activity toward other PTKs (28). Prolonged treatment with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate resulted in down-regulation of all isozymes of PKC in IC.DP cells except PKC-ζ and, suprisingly, PKC-β_{II}; 12-*O*-tetradecanoylphorbol-13-acetate did not affect the kinetics of apoptosis or its suppression by v-Abl PTK (27). Taken together, these results suggested that one signaling molecule involved in the suppression of apoptosis by v-Abl PTK is PKC and specifically, in these cells, the isozymes PKC-β_{II} and/or PKC-ζ. Having demonstrated the effects of calphostin C on v-Abl PTK-mediated suppression of apoptosis induced by cytokine deprivation, we assessed the effects of calphostin C on v-Abl PTK-mediated drug resistance in IC.DP cells treated with melphalan or hydroxyurea. IC2.9 cells (no v-Abl PTK) and IC.DP cells were treated at 32° (v-Abl PTK active) with either melphalan (10 μM) or hydroxyurea (10 mM) and calphostin C (25–250 ng/ml). Fig. 5A shows the cellular response to melphalan together with increasing concentrations of calphostin C at 72 hr. Nonspecific cytotoxicity of calphostin C was evident at concentrations above 150 ng/ml; at these concentrations of calphostin C, IC.DP cells were as sensitive to the drug as were IC2.9 cells. At concentrations of calphostin C of 150 ng/ml and below, drug sensitivity was significantly restored to IC.DP cells with active v-Abl PTK, to the extent that they were as responsive to the cytotoxic effects of melphalan as the parental IC2.9 cells. Fig. 5B shows the effect of 100 ng/ml calphostin C on IC2.9 and IC.DP cells (at this concentration there was no significant inhibition of v-Abl PTK-mediated increases in tyrosine-phosphorylated proteins after activation of v-Abl PTK) (27); there was no significant difference in the kinetics of cell death between IC2.9 cells and IC.DP cells treated with melphalan and calphostin C (Student's *t* test, $p > 0.05$). It should also be noted that IC2.9 cells were equally

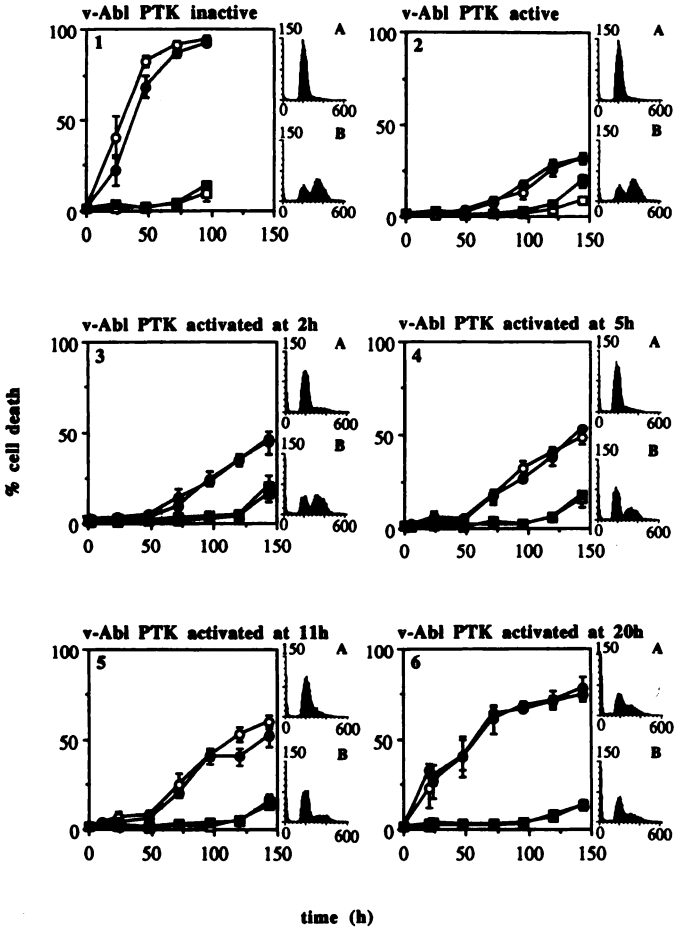

Fig. 3. Effect of cell cycle distribution on the suppression of melphalan-induced apoptosis by v-Abl PTK. IC.DP cells were separated into G_1- and S/G_2M-enriched populations by centrifugal elutriation (*1*, A and B, respectively) and treated with melphalan (10 μM). v-Abl PTK was activated at the specified times by temperature switching from the restrictive temperature (39°) to the permissive temperature (32°). Representative cell cycle distributions of melphalan-treated cells (A and B, originally enriched G_1 and S/G_2M populations, respectively) are shown for the time of activation of v-Abl PTK. Red fluorescence (575 ± 22 nm, DNA-propidium) of 10^4 cells was analyzed for each cell sample. Cell death was measured as the inability of cells to exclude trypan blue and is expressed as a percentage of the total cell count. Data points represent the means of three experiments. *Error bars*, standard errors. □, Control cells, G_1 fraction; ○, melphalan-treated cells, G_1 fraction; ■, control cells, S/G_2M fraction; ●, melphalan-treated cells, S/G_2M fraction.

sensitive to melphalan and melphalan plus calphostin C, indicating that the increase in cell death seen with IC.DP cells treated with melphalan and calphostin C did not merely reflect an additive cytotoxic effect of the two drugs. A similar restoration of sensitivity to hydroxyurea was also seen after treatment of IC.DP cells with calphostin C (Fig. 5, C and D). A leftward shift in the calphostin C concentration-response curve was noted in the hydroxyurea experiments. This is probably because we re-added calphostin C after resuspension in hydroxyurea-free medium, effectively giving these cells two exposures to "fresh" calphostin C. Although it has been suggested that calphostin C may irreversibly bind to its target, we wanted to maintain constant culture conditions similar to those used for experiments examining the sensitivity of cells to calphostin C and melphalan.

TABLE 3

Cell cycle distribution of IC.DP cells after centrifugal elutriation (0 hr) and upon activation of v-Abl PTK by temperature switching from the restrictive temperature (39°) to the permissive temperature (32°), with or without treatment with melphalan (10 μM)

Red fluorescence (575 ± 22 nm, DNA-propidium) of 10^4 cells was analyzed for each cell sample. Cell cycle histograms were analyzed using Cell Fit software and a manual program (Becton Dickinson). The results shown are the average ± standard error of three repeated experiments.

Time of switch to 32°	Distribution											
	G_1-enriched						S/G_2M-enriched					
	Control			Melphalan-treated			Control			Melphalan-treated		
	G_1	S	G_2M	G_1	S	G_2M	G_1	S	G_2M	G_1	S	G_2M
	%											
0 hr	94.3 ± 1.1	3.1 ± 0.8	2.6 ± 0.6				42.1 ± 8.6	18.9 ± 0.4	39.0 ± 8.4			
2 hr	95.5 ± 1.7	2.4 ± 0.9	2.1 ± 0.9	94.5 ± 2.9	3.0 ± 1.2	2.5 ± 1.7	43.6 ± 5.9	22.7 ± 6.6	33.7 ± 0.7	43.8 ± 8.0	22.6 ± 4.7	33.5 ± 3.2
5 hr	92.8 ± 1.0	4.6 ± 0.6	2.5 ± 0.5	93.6 ± 1.3	4.4 ± 0.8	2.0 ± 0.5	71.4 ± 1.9	8.0 ± 1.2	20.5 ± 2.2	62.6 ± 1.3	11.5 ± 2.5	25.9 ± 3.0
11 hr	80.1 ± 3.6	12.0 ± 2.4	7.8 ± 1.6	82.6 ± 2.5	13.2 ± 2.5	4.2 ± 0.9	86.7 ± 3.3	6.5 ± 1.1	6.8 ± 2.3	67.3 ± 2.4	10.6 ± 1.6	22.1 ± 2.6
20 hr	68.7 ± 1.3	14.2 ± 1.6	17.0 ± 2.6	51.5 ± 5.3	31.5 ± 4.1	17.0 ± 3.1	64.1 ± 0.2	15.8 ± 0.9	20.0 ± 1.1	54.7 ± 8.8	23.8 ± 4.6	22.2 ± 4.2

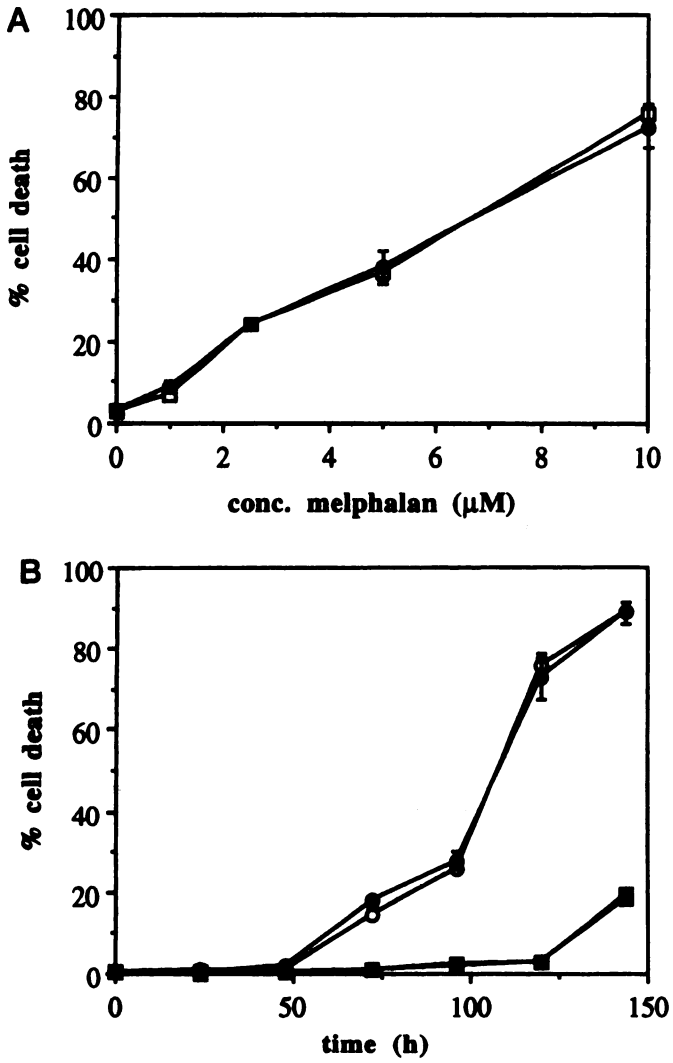

Fig. 4. A, Effect of a supraoptimal concentration of IL-3 on IC2.9 cells (no v-Abl PTK) treated at 32° with increasing concentrations of melphalan (1–10 μM). □, 50 units/ml IL-3; ●, 500 units/ml IL-3. B, Effect of a high concentration of IL-3 on the kinetics of cell death of IC2.9 cells treated at 32° with melphalan (10 μM). □, 50 units/ml IL-3, control cells; ○, 50 units/ml IL-3, melphalan-treated cells; ■, 500 units/ml IL-3, control cells; ●, 500 units/ml IL-3, melphalan-treated cells. Cell death was measured as the inability of cells to exclude trypan blue and is expressed as a percentage of the total cell count. Data points represent the means of three experiments. *Error bars*, standard errors.

Discussion

Drug resistance is a major problem in current cancer chemotherapy and is in part accounted for by classical biochemical mechanisms of intrinsic and acquired drug resistance, such as a low growth fraction of tumors, up-regulation of the drug target, decreased drug accumulation, and/or increased repair of drug-induced damage (8). Recently, a body of evidence has accumulated to suggest that resistance to cancer chemotherapy may also occur downstream of the primary drug-target interaction (and the immediate consequences of this interaction) via suppression of the engagement of apoptosis (9, 11, 12). New strategies for cancer chemotherapy should therefore focus not only on the mechanisms of cell proliferation but also on the molecular mechanisms whereby tumor cell apoptosis is suppressed.

CML is not generally considered to be a drug-resistant disease. Patients with chronic-phase CML are routinely treated with hydroxyurea, which has the palliative effect of reducing the granulocytosis in these patients. Over a period of, on average, 7 years, drug therapy ceases to be effective and an overtly drug-resistant blast crisis ensues. The use of more aggressive chemotherapeutic regimes does not, however, exterminate the Ph^+ clone (29), suggesting that the Ph^+ stem cell does have a tendency to resist treatment with currently employed chemotherapeutic agents. How is this effect achieved?

CML is characterized by the formation of the chimeric *bcr-abl* gene on the Ph chromosome, resulting in the constitutive activation of the Abl PTK. One possible explanation for the observed progression of CML from the chronic phase to blast crisis, and the resistance of the Ph^+ stem cell to drug treatment, is that the Abl PTK provides some cells with an as yet unquantified survival advantage, facilitating the accumulation of genetic mutations that precipitate blast crisis. For example, in situations where drugs induce limited cell damage, CML cells with a survival advantage may have marginally more time to repair the damage, compared with normal cells, which would rapidly engage apoptosis. This Abl-mediated cell survival advantage may only need to be a subtle effect *in vivo* to allow the gradual expansion of a drug-resistant cell population. The gradual emergence and domination of the Ph^+ clone in patients who have received chemotherapy throughout the chronic phase of CML has been previously referred to as regrowth resistance (30).

We (14) and others (15) have used cell line model systems to show that v-Abl PTK or Bcr-Abl suppresses apoptosis induced by a variety of drugs with different cellular targets. Here we have extended this work to further characterize the suppression of drug-induced apoptosis by v-Abl PTK in the IC.DP murine mast cell line (13, 22).

Exposure of cells to hydroxyurea is known to lead to the formation of DNA single-strand breaks, and melphalan induces DNA interstrand cross-links (25, 31). Using alkaline elution to quantitate the level of drug-induced DNA damage, we demonstrated that this was unaffected by v-Abl PTK activity. Melphalan enters cells via an active uptake mechanism, and it was therefore of interest that no difference in melphalan-induced DNA damage was detected at the different temperatures used (Fig. 2A). The time required for maximal uptake of melphalan into leukemia cells at 37° has been reported to be 18 min (32). In our experiments the exposure to melphalan was for 18 hr, so even at 32° one might expect there to be maximal uptake of melphalan. It is still possible that any differences in DNA damage (due to differential drug uptake) received by cells at 32° or 39° may be too small to be detected using alkaline elution. However, the results presented here are consistent with our previous observation that IC2.9 cells (without v-Abl PTK activity) at 32° certainly receive sufficient DNA damage over an 18-hr period to engage apoptosis (14). Together, these data demonstrate that cells with active v-Abl PTK receive all of the measurable drug-induced DNA damage but are unable to couple this damage to the apoptotic pathway. This observation has very important implications for the treatment of CML; drug-treated cells expressing Bcr-Abl PTK would contain damaged DNA but would be more resistant to apoptosis than their normal counterparts. Hincks *et al.* (33) showed that, when peripheral

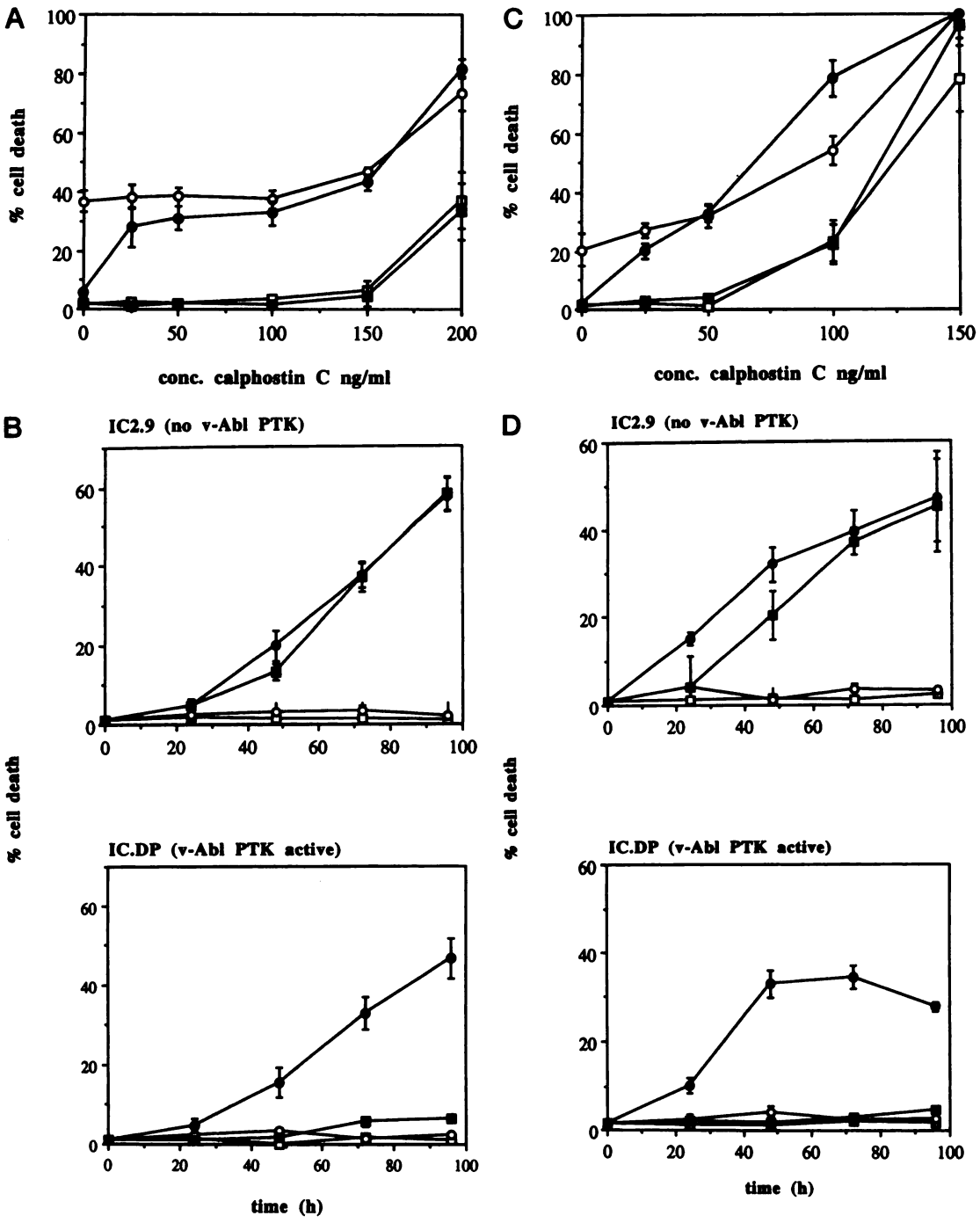

Fig. 5. A, Concentration-dependent effect of calphostin C on IC2.9 cells (no v-Abl PTK) and IC.DP cells at 32° (v-Abl PTK active) treated for 72 hr with or without melphalan (10 μM). Cell death was measured as the inability of cells to exclude trypan blue and is expressed as a percentage of the total cell count. Data points represent the means of three experiments. *Error bars*, standard errors. □, IC2.9 cells; ○, IC2.9 cells treated with melphalan; ■, IC.DP cells; ●, IC.DP cells treated with melphalan. B, Time course for the effect of 100 ng/ml calphostin C on IC2.9 cells (no v-Abl PTK) and IC.DP cells at 32° (v-Abl PTK active) treated with or without melphalan (10 μM). Cell death was measured as the inability of cells to exclude trypan blue and is expressed as a percentage of the total cell count. Data points represent the means of three experiments. *Error bars*, standard errors. □, Control cells; ○, cells treated with calphostin C; ■, cells treated with melphalan; ●, cells treated with melphalan and calphostin C. C, Concentration-dependent effect of calphostin C on IC2.9 cells (no v-Abl PTK) and IC.DP cells at 32° (v-Abl PTK active) treated for 24 hr with or without hydroxyurea (10 mM), resuspended in hydroxyurea-free medium, and treated for 48 hr with or without calphostin C. Cell death was measured as the inability of cells to exclude trypan blue and is expressed as a percentage of the total cell count. Data points represent the means of three experiments. *Error bars*, standard errors. □, IC2.9 cells; ○, IC2.9 cells treated with hydroxyurea; ■, IC.DP cells; ●, IC.DP cells treated with hydroxyurea. D, Time course for the effect of 50 ng/ml calphostin C on IC2.9 cells (no v-Abl PTK) and IC.DP cells at 32° (v-Abl PTK active) treated for 24 hr with or without hydroxyurea (10 mM) and resuspended in hydroxyurea-free medium. Cell death was measured as the inability of cells to exclude trypan blue and is expressed as a percentage of the total cell count. Data points represent the means of three experiments. *Error bars*, standard errors. □, Control cells; ○, cells treated with calphostin C; ■, cells treated with hydroxyurea; ●, cells treated with hydroxyurea and calphostin C.

blood cells from patients with chronic-phase CML were exposed to busulphan, 1% survived treatment. If these resistant cells were in some way genetically damaged but subsequently able to proliferate, they might actually contribute to the progression of CML from the chronic phase to blast crisis. One of the genes implicated in this progression is mutated *p53* (34), resulting in the loss of a G_1 checkpoint before cell division. It has recently been shown that the alkylating agent *N*-ethyl-*N*-nitrosourea causes mutations within codon 248 of *p53* (35), one of the 'hotspot' mutation regions (36) that has been found to be mutated in blast crisis (37). Thus, treatment of patients with CML with DNA-damaging drugs could, while controlling the chronic phase, potentially predispose the patients to further genetic mutation and consequent disease progression.

To further examine Abl PTK-mediated drug resistance and specifically to assess whether Abl PTK exerts its effects on drug resistance in a cell cycle-dependent manner, IC.DP cells were treated with melphalan in either the G_1 or S/G_2M phase of the cell cycle; this revealed no significant difference in the ability of v-Abl PTK to suppress apoptosis. What was also apparent from these studies was that, the longer the time period after drug addition with Abl PTK inactive (up to 11 hr), the more cell death was observed from 3 days after drug addition. Presumably, some cells reached a cell cycle-independent but irreversible 'cell death checkpoint' (38) before v-Abl PTK was activated, whereas others had not reached this death checkpoint and could be rescued.

Given that the phosphorylation patterns induced by the Bcr-Abl, v-Abl, and IL-3 PTKs show some overlap (26, 39) and that IL-3, in addition to providing a mitotic stimulus, can suppress apoptosis (13, 40), it is possible that the suppression of drug-induced apoptosis by v-Abl PTK reflects the use of the same signaling pathway elicited by IL-3. All of our earlier experiments to demonstrate the suppression of drug-induced apoptosis by v-Abl PTK were performed in cultures where the concentration of IL-3 was sufficient to stimulate cell proliferation. Despite this presence of IL-3, cells with inactive v-Abl PTK or no v-Abl PTK died by apoptosis after drug treatment, suggesting that v-Abl PTK imposed a survival advantage over and above that imparted by IL-3. To investigate the relative survival stimuli provided by v-Abl and IL-3, experiments using recombinant IL-3 at 10 times the normal cell culture concentration and a range of concentrations of melphalan were performed. IL-3 had no effect on melphalan-induced apoptosis in parental cells (no v-Abl PTK), providing corroborating evidence that the survival function of v-Abl PTK is independent of IL-3.

We previously suggested an important role for PKC in the suppression of apoptosis by v-Abl PTK (27). In IC.DP cells, activation of v-Abl PTK results in the translocation of PKC-β_{II} to the nucleus; candidate substrates for PKC within the nucleus that may be important for the regulation of apoptosis include nuclear lamins and p53 (11). Calphostin C (a relatively selective inhibitor of PKC), at concentrations that did not affect tyrosine phosphorylations, both prevented the translocation of PKC to the nucleus and restored an apoptotic response to the withdrawal of IL-3 (27). To explore the function of PKC with respect to v-Abl PTK-mediated drug resistance, calphostin C and hydroxyurea or melphalan were added to IC.DP cells with active v-Abl PTK. Calphostin C restored the drug sensitivity of cells with active v-Abl PTK to the extent that they were as sensitive as the cells containing no v-Abl PTK. Although the data described above strongly implicate PKC, we cannot rule out the possibility that calphostin C affects other cellular kinases. Nevertheless, the combination of a conventional cytotoxic agent with a downstream modifier of the Abl-mediated survival pathway may result in increased killing of leukemic cells, providing enhanced cytotoxicity to CML cells.

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