Apoptosis Is Regulated by the Rate of Glucose Transport in an Interleukin 3 dependent Cell Line

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Summary

In the absence of a survival stimulus, the interleukin 3 (IL-3)–dependent IC.DP cell line undergoes a process termed programmed cell death or apoptosis. Survival can be induced by IL-3, which can also stimulate proliferation of IC.DP cells. IC.DP cells have been stably transfected with the $p160^{v\text{-}abl}$ protein tyrosine kinase, activation of the kinase at the permissive temperature permits cell survival in the absence of IL-3 by suppression of apoptosis, although the growth factor is still required for proliferation. Both IL-3 and activation of the v-ABL tyrosine kinase stimulated glucose transport, which may in part be due to a translocation of transporters to the cell surface. Inhibition of glucose uptake markedly increased the rate of apoptosis in these cells, an effect that could be reversed by the provision of alternative energy sources such as glutamine. Growth factor– or oncogene-mediated increases in glucose uptake may therefore represent an important regulatory point in the suppression of apoptosis.

Apoptosis plays an essential role in development, both in morphogenesis and in processes such as lymphopoiesis (1, 2). In the hemopoietic system, withdrawal of hemopoietic growth factors, such as IL-3, from progenitor cells has been shown to induce apoptosis (3–5). However, the requirement for growth factors for suppression of apoptosis appears to be abrogated in some malignancies. For example, malignant cells from patients with follicular B cell lymphoma or non-Hodgkin's lymphoma exhibit an enhanced survival potential in the absence of hemopoietic growth factors (6), possibly resulting from overexpression of the *bcl*-2 gene (7, 8). Similar behavior is observed in progenitor cells from patients with chronic myeloid leukemia (9–11), which is associated with an activated form of the Abelson protein tyrosine kinase (v-ABL) (12). Such changes in the ability of hemopoietic cells to survive in adverse conditions may constitute one aspect of leukemogenesis.

The pleiotropic effects of growth factors, which include promotion of progenitor cell proliferation and development, complicate the assessment of the molecular mechanisms of apoptotic suppression. For this reason we have studied such mechanisms using the IL-3–dependent IC.DP cell line, which was generated from the IC2.9 mast cell line (13) by transfection with a mutant *v-abl* gene encoding a temperature-sensitive protein tyrosine kinase (PTK)[1] (14). Withdrawal of IL-3 from IL-3–dependent cell lines induces a rapid decrease in metabolism of glucose (15, 16), which nuclear magnetic resonance (NMR) analysis has indicated to occur primarily via anaerobic glycolysis (17). The uptake of glucose into the cell represents a key point in the regulation of its metabolism, and is known to be stimulated by IL-3 and by other hemopoietic growth factors (15, 16, 18–20). In view of the abrogation of the survival requirement for IL-3 by activation of v-ABL, we assessed the effects of v-ABL activity on glucose uptake to determine the relationship, if any, between glucose transport and apoptotic suppression. We have previously shown that activation of v-ABL suppresses apoptosis and thus prolongs the survival, but does not allow the proliferation, of IC.DP cells in the absence of growth factor (21, 22). Here we demonstrate that v-ABL activation suppresses the onset of apoptosis in IC.DP cells via a stimulation of glucose uptake.

Materials and Methods

Cell Culture and Reagents. IC.DP cells were maintained in Fischer's medium supplemented with 10% (vol/vol) horse serum and 3% (vol/vol) IL-3–containing X63-Ag-653 cell-conditioned medium as previously described (21, 22).

Detection of Phosphorylation of the $p160^{v\text{-}abl}$ Tyrosine Kinase. ^{32}P-labeled cell lysates were immunoprecipitated with polyclonal antibodies against c-ABL (Cambridge Bioscience, Cambridge, UK), followed by SDS/polyacrylamide gel electrophoresis and autoradiography using previously described methods (21, 22).

Detection of GLUT1 by Western Blotting. SDS/polyacrylamide electrophoresis and Western blotting were performed as described

[1] *Abbreviations used in this paper:* CB, cytochalasin B; DCB, dihydrocytochalasin B; 2-DOG, 2-deoxy-D-glucose; PBST, PBS with Tween; PTK, protein tyrosine kinase.

previously (23). After blotting the nitrocellulose membrane blocked with PBS containing 5% nonfat dry milk powder for ≥1 h at room temperature and then incubated with PBS containing 1% milk powder, 0.05% (vol/vol) Tween 20, and 1 μg/ml of rabbit antibody against GLUT-1 (24) for ≥1 h at room temperature. After washing twice with PBS containing 0.05% (vol/vol) Tween 20 (PBST) for 15 min, the membrane was incubated with PBST containing 1% milk powder and a 1:10,000 dilution of horseradish peroxidase–conjugated donkey antibody against rabbit IgG (Amersham International, Bucks, UK) for ≥1 h at room temperature. After 2 washes with PBST for 15 min, the blot was rinsed with PBS and 0.125 ml/cm² of electrochemiluminescence (ECL) solutions mixture (Amersham International) was added. The pattern of fluorescence on the membrane was recorded using Kodak X-OMAT-AR film.

2-Deoxy-D-Glucose (2-DOG) Uptake Assay. Cells were taken from mid log-phase cultures grown for 18 h at 39°C to ensure inactivation of v-ABL (21, 22). They were then washed three times over a period of 2 h at 39°C in Fischer's medium containing 5 mM Hepes, pH 7.4, to remove IL-3. For 2-DOG uptake assays, cells were rapidly centrifuged and resuspended in ice-cold PBS, pH 7.3. They were then kept on ice for <30 min before assaying the uptake of [³H]2-DOG (Amersham International). Transport was measured at 37°C with 1 mM 2-DOG, and an uptake period of 1 min, using the oil-stop assay previously described (15).

Confocal Immunofluorescence Microscopy. Cells were prepared for microscopy by centrifugation onto glass slides using a Shandon cytospin II centrifuge for 3 min at 200 *g*. After air drying for ≥1 h, the cells were fixed and permeabilized with acetone for 3 min at room temperature. Slides were stained by incubation for 30 min with affinity-purified rabbit antibody against the COOH terminus of GLUT1 (24) (10 μg/ml in PBS containing 10% FCS and 0.1% sodium azide) followed by FITC-conjugated goat anti–rabbit IgG (Pierce & Warriner, Chester, UK). After mounting in 90% glycerol containing 25 mg/ml 1,4-diazobicyclo-(2,2,2)-octane (25) (Sigma Chemical Co., Poole, UK) slides were examined using a microscope (Zeiss, Cambridge, UK) equipped with a laser (model MRC 600; Bio-Rad Laboratories, Richmond, CA) scanning confocal unit.

Assay for Apoptosis. IC.DP cells were cultured at 39°C overnight to ensure inactivation of v-ABL. They were then washed three times to remove IL-3 before resuspension at a concentration of 10^6/ml in DME (without L-glutamine; ICN Flow, Thames, UK), plus the additives indicated. After incubation for 18 h at 39 or 32°C, apoptosis was measured by acridine orange staining to assess nuclear morphology, as previously described (26).

Results and Discussion

Treatment with IL-3 or Activation of v-ABL Stimulates Glucose Transport in IC.DP Cells. IC.DP cells constitutively express the $p160^{v\text{-}abl}$ tyrosine kinase (Fig. 1 *A*), which is active at 32°C (permissive temperature) but not at 39°C (restrictive temperature). To study the effects of this PTK on glucose transport, we assayed [³H]2-deoxyglucose uptake. This was potently inhibited by cytochalasin B (CB; see also Fig. 3 *A*), indicating that it was catalyzed by a member(s) of the GLUT family of passive sugar transporters (27). Western blotting of IC.DP cell lysates using antibodies raised against the COOH terminus of GLUT1 (24) showed that this transporter isoform was present in cells grown either at 32°C or at 39°C, with (see Fig. 1 *D*) or without IL-3 (see Fig. 1 *B*). In contrast, neither GLUT2, GLUT3, nor GLUT4 was de-

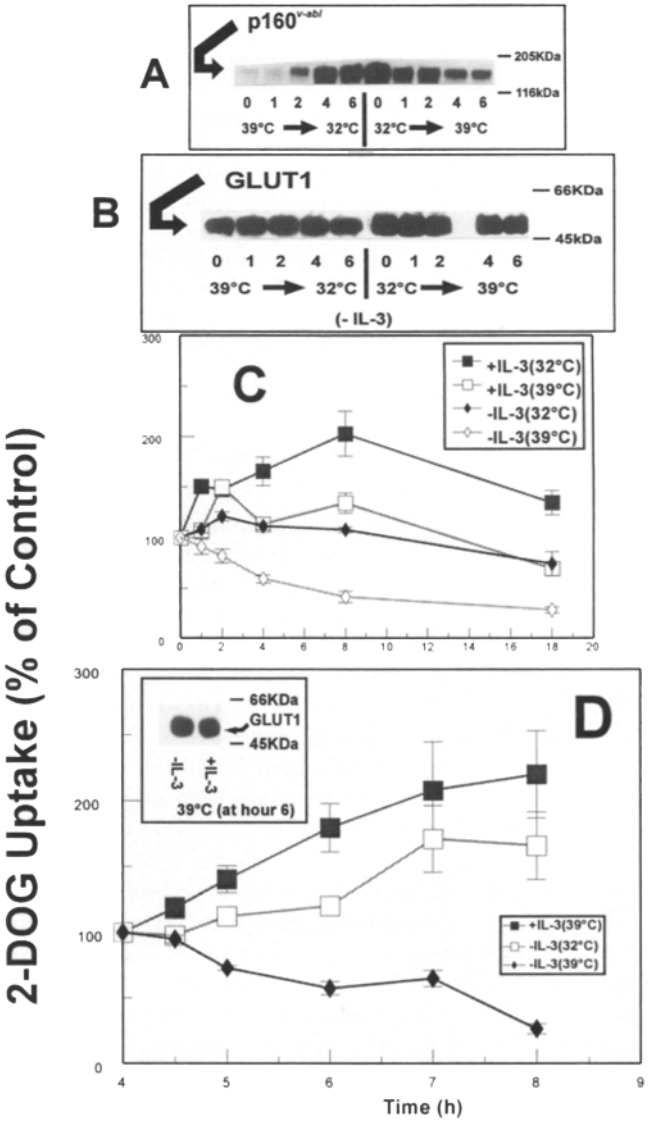

Figure 1. Effects of v-ABL activation and IL-3 on glucose transport in IC.DP cells. (*A*) Phosphorylation of $p160^{v\text{-}abl}$ was measured in IC.DP cells that had been maintained for 18 h at the restrictive (39°C) or permissive (32°C) temperatures for v-ABL activity, and then switched to the alternate temperature for the times shown. (*B*) Total cellular GLUT1 content was measured in IC.DP cells that had been treated as in *A*. Results shown are from one representative experiment of three. (*C*) Cells were incubated for a further period at 39°C, or treated with purified recombinant IL-3 (100 U/ml; a gift from Dr. J. Schreurs, DNAX, Palo Alto, CA) and/or switching the temperature to 32°C. The uptake of 2-DOG was measured at the times indicated. The results shown are the mean ± SEM of three experiments, and are expressed as a percentage of the uptake measured at 0 h (i.e., 2 h after washing to remove IL-3 was initiated). The latter value was 2.36 nmol/min/10^6 cells. (*D*) Cells were incubated for a further 4 h after "0 h" at 39°C without IL-3 and then incubated with or without IL-3 at the temperatures indicated for a further period and the uptake of 2-DOG was measured at various times. Results (mean ± SEM of three experiments) are expressed as a percentage of the uptake at 0 h (i.e., 6 h after washing to remove IL-3 was initiated). The latter value was 0.80 nmol/min/10^6 cells. The *inset* shows the GLUT1 content of the cells at 39°C (at "6 h") with or without IL-3.

tectable using antibodies specific for these isoforms (data not shown).

When cells that had been grown overnight at 39°C in the presence of IL-3 were subsequently deprived of the growth factor and maintained at 39°C, 2-DOG uptake rates declined substantially relative to those for cells maintained in the presence of IL-3 (Fig. 1 *C*). Readdition of IL-3 to cells deprived of the growth factor for 4 h caused a rapid return of uptake rates to the original levels, with a $t_{0.5}$ of about 1.5 h (Fig. 1 *D*). However, cells incubated at 32°C did not exhibit significant loss of transport activity in the absence of IL-3 (Fig. 1 *C*). This finding suggests that activation of v-ABL can partly mimic the effect of IL-3 on glucose transport. Confirmation of this suggestion was provided by the observation that switching the temperature to 32°C for cells deprived of IL-3 for 4 h at 39°C not only prevented any further decline of transport activity over the subsequent 4 h, but in fact increased uptake rates by ~50% over this period (Fig. 1 *D*).

To further characterize the transport activation induced by activation of the kinase, initial rates of sugar uptake were measured over a range of 2-DOG concentrations. Estimates of the V_{max} and K_m were obtained using nonlinear curve fitting of the data (Enzfitter; Elsevier-Biosoft, Cambridge, UK; data not shown). A comparison was made of cells maintained at 39°C for 6 h in the absence of IL-3 with cells maintained under these conditions for 4 h and then switched to 32°C for 2 h. The results indicated that activation of v-ABL significantly increased the V_{max} for transport, from 1.52 ± 0.09 to 2.48 ± 0.15 nmol/min/10^6 cells (mean ± SEM, $n = 3$, $p < 0.01$). Also, there was a decrease on the K_m, which was 4.71 ± 0.28 mM for the cells maintained at 39°C compared with 2.78 ± 0.30 mM (mean ± SEM, $n = 3$, $p < 0.01$) for the cells switched to 32°C.

IL-3 treatment or v-ABL Activation Alters Subcellular Distribution of the GLUT1 Glucose Transporter in IC.DP Cells. Chronic stimulation of glucose uptake by mammalian cells in response to serum, IL-1, or oncogenes such as the v-SRC PTK that is related to v-ABL, is mediated by an increase in the total cellular content of glucose transporters (28). In contrast, the acute increase in sugar transport seen in many cell types after exposure to serum, stress, or insulin appears to result primarily from translocation of the GLUT1 and/or GLUT4 transporter isoforms from an intracellular compartment to the plasma membrane (27–30). To ascertain the origin of the increase in transport V_{max} induced in IC.DP cells by activation of v-ABL, quantitative Western blotting of GLUT1 was performed as previously described (23). In no case did we observe changes in the GLUT1 content of the cells as a consequence of switching temperature from 39 to 32°C or from 32 to 39°C (see Fig. 1 *B*), indicating that the action of v-ABL on glucose transport is not mediated by changes in the total cellular content of transporters. Furthermore, confocal immunofluorescence microscopy showed not only that IC.DP cells contain a cytoplasmic, vesicular pool of GLUT1, but also that when IL-3–deprived cells were switched from 39 to 32°C, or IL-3 was added, there was a significant increase in the cell surface staining of GLUT1 (Fig. 2). This finding suggests that the effects both of IL-3 and of v-ABL activation on transport are at least in part mediated by transporter translocation from an intracellular pool to the cell surface, although v-ABL also appears to increase the apparent affinity of the transporter for substrate. Any role of change in the total GLUT1 levels present in these cells is excluded by the results of quantitative Western blotting experiments (see Fig. 1, *B* and *D*). In addition, the presence of the protein synthesis inhibitor cycloheximide (up to 100 μM) had no significant effect on the changes in transport activity brought about by activation of v-ABL or addition of IL-3 to IL-3–deprived cells (data not shown). Furthermore the kinetics of the transport stimulation ($t_{1/2} \simeq 1$–2 h) resemble those for stress- and serum-induced GLUT1 translocation in other cell types that, similarly, are independent of protein synthesis (29, 30).

Inhibition of Glucose Transport Induces Apoptosis in IC.DP Cells, an Effect That Can Be Reversed by Provision of Alternative Energy Sources. The observations described above suggested that suppression of apoptosis upon activation of v-ABL might be mediated partly via its effect upon sugar uptake by the IC.DP cells. To investigate this hypothesis further, we examined the effect of the potent inhibitor of glucose transport, CB. At a concentration of 10 μM (which can be calculated to reduce the rate of glucose transport by 17-fold at the concentration of extracellular glucose used in these experiments), this compound caused a ≥fourfold increase in the rate of apoptosis of IL-3–deprived cells in which v-ABL had been activated by incubation at 32°C (Fig. 3, *B* and *C*). The effect of CB on apoptosis was dose dependent, a twofold increase being observed at concentrations (0.1–0.5 μM) equivalent to the K_i for inhibition of GLUT1-mediated transport (Fig. 3 *B*) (28). For cells incubated at 39°C, where the rate of apoptosis was already elevated as a result of inactivation of v-ABL, addition of CB increased apoptosis rates still further (Fig. 3 *C*). The involvement of transport inhibition rather than cytoskeletal disruption in this phenomenon was confirmed by comparing the effect of dihydrocytochalasin B (DCB) with CB. DCB is a less potent inhibitor of transport (See Fig. 3 *A*), but resembles CB in binding tightly ($K_d \simeq 10$ nM) to microfilaments (31). Although DCB did increase apoptosis rates to some extent, its effect was much smaller than that of CB at all concentrations tested (Fig. 3 *B*). The mode of cell death induced by CB was confirmed to be apoptosis using flow cytometric analysis (data not shown) (32).

Additional support for the hypothesis that deprivation of glucose, a major energy source for hemopoietic cells, plays a part in the induction of apoptosis by CB or by IL-3 deprivation, was provided in several ways. First, it was found that the effect of CB on the rate of apoptosis could be partly reversed by the provision of L-glutamine or pyruvate as alternative energy sources (Fig. 3 *C*). These additives, in particular L-glutamine, also significantly suppressed the apoptosis produced by incubation of IL-3–deprived IC.DP cells at 39°C (Fig. 3 *C*). Second, we found that another competitive inhibitor of transport, the membrane-impermeant disaccharide maltose (28), could also induce apoptosis. Its IC_{50} for inhibition of 1 mM 2-DOG uptake by IC.DP cells was about

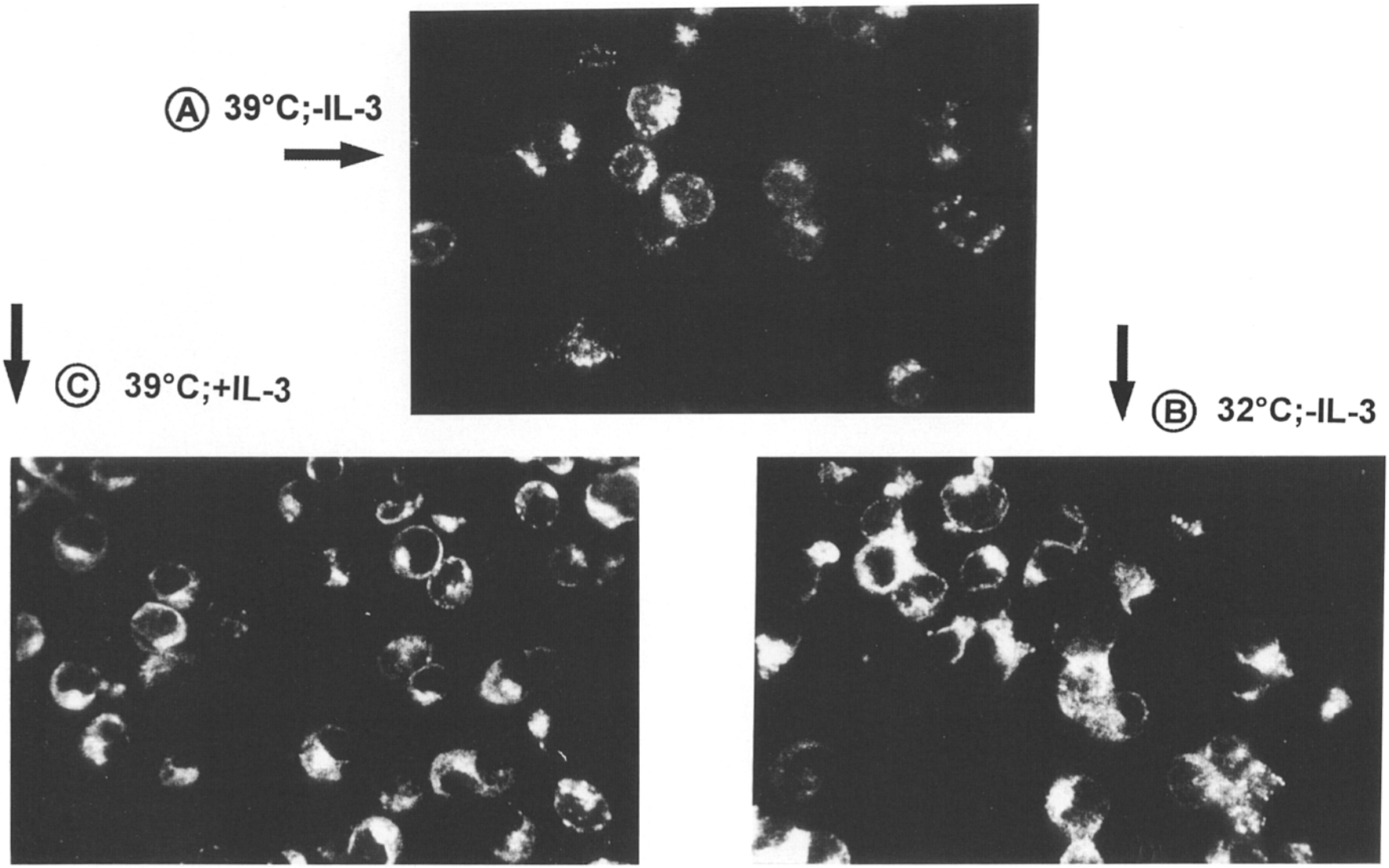

Figure 2. Effect of IL-3 and temperature on the subcellular distribution of GLUT1. IC.DP cells were grown overnight at 39°C to inactivate v-ABL and then deprived of IL-3 for a total of 6 h at 39°C, as described in Fig. 1 *C*. They were then incubated for a further 2 h without IL-3 at 39°C (*A*) or at 32°C (*B*), or were treated with IL-3 (100 U/ml) at 39°C for 2 h (*C*). The subcellular distribution of GLUT1 was then examined by confocal microscopy.

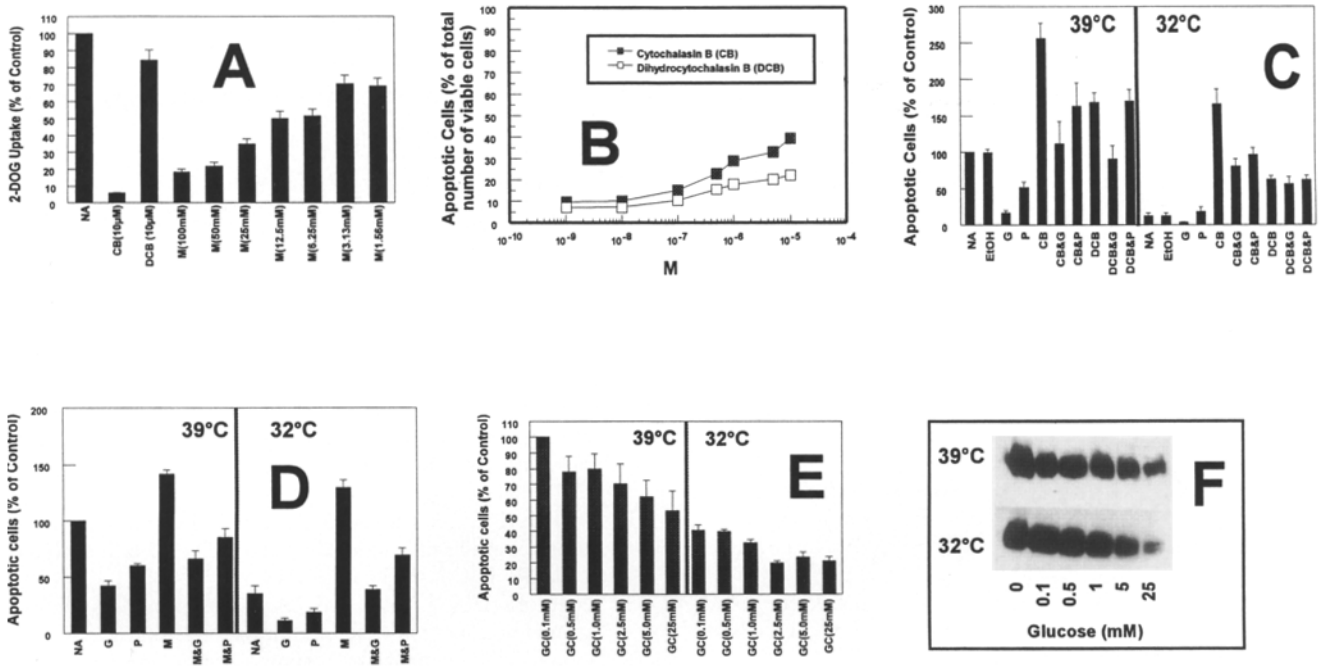

Figure 3. The effect of glucose transport inhibition and of alternative energy sources on apoptosis in IC.DP cells. (*A*) The effect of CB, DCB and maltose (M) on 2-DOG uptake by IC.DP cells grown at 32°C was assessed by adding the compounds as indicated immediately before assay of transport. Results (mean ± SEM, $n = 3$) are expressed as a percentage of the uptake seen in the absence of any additive (NA), which was 2.18 nmol/min/10^6 cells. (*B*) Dose-response curves for the effect of CB and DCB on the rate of apoptosis in IC.DP cells grown at 32°C (mean ± SEM, $n = 3$). (*C*) The effect of 2 mM L-glutamine (G) or 10 mM pyruvate (P) on the rate of CB (10 μM) induced apoptosis. Results (mean ± SEM, $n = 3$) are expressed as a percentage of the number of apoptotic cells per 100 viable cells seen at 39°C without any additive (*NA*). Ethanol (EtOH) was used as the carrier for CB and DCB. (*D*) The effect of 2 mM L-glutamine (G) or 10 mM pyruvate (P) on the rate of 20 mM maltose-induced apoptosis in IC.DP cells. Results (mean ± SEM, $n = 3$) are expressed as a percentage of the number of apoptotic cells/100 viable cells seen at 39°C without any additive (NA). (*E*) The effect of extracellular glucose concentration on the rate of apoptosis in IC.DP cells. Cells were treated as in (*C*) and (*D*) except that incubations were in PBS, pH 7.3, containing 0.1% (wt/vol) BSA and the concentration of glucose indicated. Results (mean ± SEM, $n = 3$) are expressed as a percentage of the number of apoptotic cells present after incubation in 0.1 mM glucose (GC) at 39°C, which was 95% of the number of viable cells. (*F*) The effect of glucose starvation on expression of GLUT1 in IC.DP cells. Results shown are from one representative experiment of three.

10 mM (Fig. 3 *A*). Addition of 20 mM maltose (but not 20 mM sucrose, which does not inhibit transport, data not shown) inhibited the suppression of apoptosis produced by activation of v-ABL in IL-3–deprived IC.DP cells, thereby increasing the rate of apoptosis to a level similar to that seen at 39°C (Fig. 3 *D*). Once again, provision of L-glutamine or pyruvate as alternative energy source partially overcame the effect of maltose (Fig. 3 *D*). Finally, we found that directly decreasing the glucose supply to the cells, by lowering the sugar concentration in the medium, also increased the rate of apoptosis in IL-3–deprived cells, both at 32 and 39°C (Fig. 3 *E*). Much more severe restriction of glucose supply was required to induce apoptosis in such experiments than had been expected from the effects of maltose or CB reported above. However, the relatively small effect on apoptosis observed in a decrease between 25 and 2.5 mM glucose may be due to the fact that we observed an increase in GLUT1 expression upon glucose deprivation (see Fig. 3 *F*), as has previously been described elsewhere (33–37), which will presumably lead to greater glucose uptake.

Taken together, our results indicate that the supply of energy in the form of glucose or other substances directly influences the rate of apoptosis in IC.DP cells. We suggest that one of the criteria that hemopoietic cells need to meet in order to avoid apoptosis is to maintain a favorable energy balance. Should the latter fall below a certain crucial point, the preexisting program for apoptosis will be executed. Oncogene and cytokine stimulation of glucose transport therefore represents a regulatory control point in the suppression of apoptosis. We do not suggest that maintenance of a favorable energy balance is the sole regulatory point governing apoptosis in hemopoietic cells; cytokines and other proteins such as bcl-2 are likely to elicit a pleiotropic set of effects to suppress apoptosis. However, our data do indicate one aspect of cellular metabolism that may be disregulated in multistage leukemogenesis.

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