Cellular Signaling Events Elicited by v-abl Associated with Growth Factor Independence in an Interleukin-3-dependent Cell Line*

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A temperature-sensitive mutant of the v-abl oncoprotein has previously been shown to have markedly reduced tyrosine protein kinase activity in interleukin 3 (IL-3)-dependent cells grown at restrictive (39 °C), compared to permissive (32 °C) temperatures. Transformation of this mutant v-abl into the IC2.9 cell line, generated the IC.DP subclone which was dependent on IL-3 for survival at 39 °C, but not at 32 °C. Furthermore, IC.DP cells cultured at 32 °C exhibited IL-3-independent thymidine incorporation, which was not apparent at 39 °C. Switching cells from the restrictive to the permissive temperature resulted in an increase in cellular inositol-1,4,5-trisphosphate, choline phosphate and diacylglycerol levels in the IC.DP cell line. These increases were only observed after a lag period of 4 h. Within 2 h of switching, IC.DP cells previously maintained at 32 to 39 °C, there was a significant decrease in all three metabolites. Temperature switches had no effect upon these metabolites in the parent IC2.9 cell line. Down-regulation of protein kinase C inhibited v-abl-stimulated DNA synthesis in IC.DP cells cultured at 32 °C. IC.DP cells cultured at 32 °C were found to have a constitutively activated Na+/H+ antiport, although this activation was inhibited by the down-modulation of protein kinase C. These data indicate a role for phospholipid hydrolysis and protein kinase C activation in V-ABL-mediated abrogation of IL-3 dependence.

The abl oncogene product is one of several tyrosine kinases that can transform hemopoietic cells in vitro and stimulate their autonomous proliferation (Cook et al., 1985; Mathey et al., 1986; Pierce et al., 1986). It was first identified as being responsible for the transforming activities of the Abelson murine leukemia virus, and there is now considerable evidence to suggest that activated abl genes have an important role in the initiation of human leukemias. A chromosomal translocation which occurs in over 95% of patients with chronic myeloid leukemia generates a chimeric bcr/abl gene. This encodes the p210bcrc/abl protein (Shitivelman et al., 1985), which may have a causal role in leukemia (Daley et al., 1990, 1991; Elefanty et al., 1990; Kellieher et al., 1990). The c-abl gene also encodes a protein tyrosine kinase, which in the context of the p210bcrc/abl fusion protein, resembles the transforming p160c-abl gene product in exhibiting deregulated tyrosine kinase activity (Clark et al., 1987; Kellieher et al., 1990; Lugo et al., 1990).

While the biological effects of p210bcrc/abl are becoming clearer, there is, as yet, little known about the biochemical effects of the abl gene product.

The mechanisms whereby v-abl and v-src stimulate cellular transformation may be related, since both belong to the src family of homologous cytoplasmic, non-receptor tyrosine kinases. The activities of v-src have been more extensively studied, and some of the biochemical events elicited by activated src tyrosine kinase include its autophosphorylation, association with signal transducing proteins containing src homology 2 domains (e.g. phosphatidylinositol-phospholipase Cγ and phosphatidylinositol-3-kinase) and stimulation of phospholipid hydrolysis to generate second messengers e.g. sn-1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). In addition, v-src activates the ras-1 serine/threonine kinase (Morrison et al., 1988; Qureshi et al., 1991), through a mechanism that may involve activation of ras and tyrosine phosphorylation of the ras GTPase activating protein, ras-GAP (Brott et al., 1991; Moran et al., 1991).

To investigate the effects of v-abl, a number of growth factor-dependent hemopoietic cell lines have been infected with Abelson murine leukemia virus, which can abrogate their growth factor requirements, and lead to autonomous proliferation without autocrine growth factor production (Cook et al., 1985; Mathey et al., 1986; Pierce et al., 1986). Maintenance of IL-3 independence has been shown to require the continuous function of v-abl by the preparation of temperature-dependent mutants of the v-abl tyrosine kinase (Kipreos et al., 1987). At the permissive temperature of 32 °C, these temperature-sensitive cell lines have an important role in the initiation of human leukemias. A chromosomal translocation which occurs in over 95% of patients with chronic myeloid leukemia generates a chimeric bcr/abl gene. This encodes the p210bcrc/abl protein (Shitivelman et al., 1985), which may have a causal role in leukemia (Daley et al., 1990, 1991; Elefanty et al., 1990; Kellieher et al., 1990). The c-abl gene also encodes a protein tyrosine kinase, which in the context of the p210bcrc/abl fusion protein, resembles the transforming p160c-abl gene product in exhibiting deregulated tyrosine kinase activity (Clark et al., 1987; Kellieher et al., 1990; Lugo et al., 1990).

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EXPERIMENTAL PROCEDURES

Cell Culture—Cells were routinely cultured in Fischer's medium, supplemented with 10% horse serum and 3% of medium conditioned by the X63-Ag-653 cell line transfected with the IL-3 gene (mIL-3 CMV) (Karasuyma and Melchers, 1982), and were maintained at 37 °C in a gassed incubator at 5% CO2. Experiments investigating

The abbreviations used are: DAG, sn-1,2-diacylglycerol; IP3, inositol 1,4,5-trisphosphate; IL-3, interleukin-3; PKC, protein kinase C; BCECF, bis(carboxyethyl)-carboxyfluorescein; TPA, 12-O-tetradecanoylphorbol-13-acetate; 5-MNIA, 5-N-(methyl-N-isobutyl)-amiloride.

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the effects of temperature switches, cells were generally maintained for the preceding 18 h at 39 or 32 °C.

**Measurement of Expression and Phosphorylation of V-ABL**—Expression of V-ABL was detected by Western blotting using a monoclonal anti-abl antibody (Oncogene Science). Cells maintained for 18 h at either 39 or 32 °C were lysed as described below. Identical amounts of cellular protein were then separated by SDS-polyacrylamide gel electrophoresis before transferring onto nitrocellulose and processing as described previously (Kan et al., 1992).

Phosphorylation of V-ABL was detected by immunoprecipitation. Cells (3 x 10⁶/ml) were labeled in phosphate free Dulbecco's modified Eagle's medium, supplemented with 3% dialyzed mIL-3 CM, 5% dialyzed horse serum, and 100 pCi/ml [:³²P]orthophosphate, and incubated for 18 h at 39 or 32 °C. Aliquots (1 ml) were then transferred at various time points to the alternative temperature. Control cells, i.e. time 0 h, were kept at 39 or 32 °C throughout, so that all cells were subjected to an equivalent labeling period. Cells were harvested by centrifugation, lysed by resuspending them in 0.5 ml of ice-cold lysis buffer (50 mM Tris acetate buffer, pH 7.5, 1 mM EDTA, 1 mM EGTA, 120 mM NaCl, 1 mM NaN₃, 50 mM NaF, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml benzamidine, 10 μg/ml antipain, and 1% (v/v) Nonidet P-40) and kept on ice for 30 min. Lysates were cleared by centrifugation and then incubated overnight with 1 μg/ml rabbit polyclonal anti-c-ABL antibody (Oncogene Science) and 30 μl/ml of protein G-agarose (Calbiochem), at 4 °C. Immunoprecipitates were collected by centrifugation and washed (four times) with lysis buffer, before being resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography.

Tyrosine phosphorylation of intracellular proteins was analyzed using immunoblotting with monoclonal anti-phosphotyrosine antibody (UBI). Proteins were resolved by SDS-polyacrylamide gel electrophoresis, and Western blotting was carried out as above except that membranes were blocked for 3 h with phosphate-buffered saline containing 2% bovine serum albumin (Fraction V).

**Measurement of Growth Characteristics**—Cellular viability was determined by trypan blue exclusion. Cells were cultured at 39 °C overnight in IL-3 containing medium, washed twice to remove the IL-3, and then cultured at 1 x 10⁶ cells/ml in Fischer's medium supplemented with horse serum (10%). Cells were then incubated at either 32 or 39 °C and viability determined at set time points.

[^3H]Thymidine incorporation was used as a measure of DNA synthesis. Cells were prepared as above and then plated at 1 x 10⁶ cells/ml in a total volume of 100 μl, with appropriate additives. After 16 h, [methyl-[^3]H]thymidine (1 μCi/well) was added, and 4 h later incorporation into acid-insoluble material was assayed (Wetton et al., 1988a). The recombinant murine IL-3 added to pHi and [:³²P]orthophosphate was used as a measure of DNA replication. After 16 h, [:³²P]thymidine (1 μCi/well) was added, and 4 h later incorporation into acid-insoluble material was assayed (Bligh and Dyer, 1959). Protein A was then precipitated from the cell lysates with trichloracetic acid, and the radioactivity associated with the precipitate was determined by liquid scintillation counting.

**Determination of Intracellular pH (pHₐ)**—Cells were cultured as described above, except that bicarbonate-buffered Fischer's medium was replaced by HEPES-buffered (25 mM) Fischer's, pH 7.2. Cells were incubated at either 32 or 39 °C as described in the relevant figure legends. pHₐ was then determined at 32 or 39 °C as appropriate using the fluorescent indicator bis-(carboxyethyl)-carboxyfluorescein (BCECF, Calbiochem) (Tsien et al., 1982), as described previously (Valiance et al., 1990).

**RESULTS**

v-ABL Expression and Activity.—The IC.DP cell line expresses a temperature-sensitive v-ABL protein tyrosine kinase. Fig. 1A confirms that the V-ABL protein is expressed in these cells and that its expression is not temperature dependent, with equal levels of the protein being observed at 39 and 32 °C. Previously, the temperature-sensitive v-ABL has been shown to undergo tyrosine phosphorylation upon switching...
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from the restrictive (39 °C) to the permissive (32 °C) temperature (Kipreos and Wang, 1988). Fig. 1B confirms that V-ABL is phosphorylated within 1 h of a temperature switch from 39 to 32 °C, with phosphorylation reaching a new steady state at 4 h. The phosphorylation of the DP p160°mut mutant is reversible, since switching cells from 32 to 39 °C results in a rapid dephosphorylation of V-ABL (Fig. 1C).

Immunoblotting experiments with anti-phosphotyrosine antibodies on whole cell lysates demonstrate that a temperature switch to the permissive temperature results in an increase in tyrosine phosphorylation of several cellular proteins. Similarly, the reverse switch resulted in a rapid fall in protein tyrosine phosphate levels. In the parent cell line, no changes in phosphotyrosine levels were observed with either temperature switch. One of the proteins which is tyrosine phosphorylated on switching from 39 to 32 °C has a molecular mass of 160 kDa which corresponds to the molecular mass of the V-ABL protein. Thus, at 32 °C the v-abl tyrosine kinase is active, whereas at 39 °C, this enzymatic activity is severely depleted.

Growth Characteristics of the IC.DP Subclone—In the absence of IL-3, IC2.9 cells rapidly lost viability, at both 39 and 32 °C (Fig. 2A). However, IC.DP cells cultured at 32 °C survived and proliferated in the absence of IL-3, although viability was lost at 39 °C (Fig. 2B). The IL-3 independence of IC.DP cells cultured at 32 °C was reversible; switching cells to 39 °C resulted in a loss of viability which occurred at the same rate as observed in IC2.9 cells.

A marked increase in IL-3-independent [3H]thymidine incorporation was observed when IC.DP cells were cultured at 32 °C (see Fig. 2D). This effect was absent in IC2.9 cells, and addition of IL-3 (100 units/ml) to IC.DP cells at 32 °C resulted in levels of DNA synthesis similar to those observed at 39 °C, (Fig. 2, C and D). Thus, activation of the v-abl tyrosine kinase abrogates the IL-3 dependence of the IC.DP subclone.

Having confirmed the expression of a temperature-sensitive p160°mut gene product in IC.DP cells, we examined the intracellular signaling events that might be associated with their IL-3-independent survival and proliferation when cultured at the permissive temperature for V-ABL activity.

Effect of V-ABL on Phospholipid Signaling Pathways—In order to determine the mechanism whereby V-ABL abrogates the IL-3 dependence of IC.DP cells, we have assessed the effects of V-ABL upon the breakdown of both phosphatidylinositol-4,5-bisphosphate (Ptd-Ins4,5-P2) and phosphatidylcholine.

Fig. 3 illustrates the effects of temperature switches upon levels of PI(3) (a product of Ptd-Ins4,5-P2 hydrolysis) in the IC.DP subclone. Cells were maintained at 39 or 32 °C for 18 h before IL-3 was removed, and cells were switched from 39 to 32 °C or vice versa, for the times shown. Temperature switches were found to have no effect upon mass PI(3) levels in IC2.9 cells in the absence of IL-3. IC2.9 cells maintained at 39 °C for 18 h contained 0.43 ± 0.13 pmol (n = 12) pmol of IP3/106 cells compared to 0.48 ± 0.13 pmol (n = 12) in cells at 32 °C. However, when IC.DP cells were shifted from the restrictive to the permissive temperature, there was a 5.55-fold increase

Fig. 2. The effect of temperature on cellular viability (A and B) and [3H]thymidine incorporation (C and D) in IC2.9 and IC.DP cells. Cell viability was assessed for IC2.9 (A) and IC.DP (B) at both 39 °C (squares) and 32 °C (circles) in the presence (open symbols) and absence (closed symbols) of IL-3. [3H]Thymidine incorporation was determined for IC2.9 (C) and IC.DP (D) at 39 °C (filled bars) and 32 °C (hatched bars). Results shown are mean of triplicates from a representative of four experiments. Standard deviation values did not exceed 10% of the mean value of any of the data points shown.

Fig. 3. The effect of temperature switch upon mass inositol-1,4,5-trisphosphate levels in IC.DP cells. IC.DP cells were maintained at 39 or 32 °C for 18 h prior to washing to remove IL-3 and incubating for 3 h before switching to 32 °C (squares) or 39 °C (circles), respectively, for the times indicated. Mass levels of inositol 1,4,5-trisphosphate were determined and corrected for cell number before expressing as a percent of control at time 0 h. Results shown are mean (n = 4) with S.E. values not exceeding 10%. Control values were IC.DP at 39 °C for 18 h, 0.41 ± 0.01 pmol/106 cells, IC.DP cells at 32 °C, 3.8 ± 1.4 pmol/106 cells.
in mass IP, following a lag phase of 4 h (see Fig. 3). Conversely, switching cells from 32 to 39 °C resulted in a rapid fall in mass IP, which returned to the levels observed in cells cultured at 39 °C within 4 h. Activation of the v-abl tyrosine kinase therefore stimulates a reversible rise in mass IP levels.

Similar experiments were carried out to examine the effects of temperature switches upon phosphatidylcholine breakdown (Fig. 4). The results presented indicate that [3H]choline phosphate levels remained constant in the parent cell line, but as was the case with IP, levels, activation of the v-abl tyrosine kinase was found to stimulate an increase in [3H]choline phosphate levels after an initial lag of 4 h (Fig. 4A). This increase was 1.98 ± 0.15-fold (mean ± S.E. n = 5) after 18 h. Switching cells to the restrictive temperature resulted in a rapid fall in [3H]choline phosphate levels. Measurement of [3H]choline showed that a switch from the restrictive to the permissive temperature resulted in a decrease in the level of this metabolite in the IC.DP subclone, while the reverse switch resulted in a corresponding increase (Fig. 4B). Temperature shifts had no effect upon the levels of [3H]choline in the IC2.9 cell line.

Measurement of [3H]glycerophosphocholine levels illustrated that they were temperature sensitive in both the IC2.9 and IC.DP cell lines and were not specifically affected by the activation of the v-abl tyrosine kinase (Fig. 4C). Switching cells from 39 to 32 °C resulted in a decrease in [3H]glycerophosphocholine levels in both IC2.9 and IC.DP cells, with the reverse switch stimulating a similar percentage increase. These changes would therefore appear to be dependent upon temperature rather than activation of V-ABL.

These experiments suggest that the temperature-induced activation of V-ABL results in an increase in phospholipase C-mediated breakdown of phospholipids. Since DAG is also formed as a product of both inositol lipid and phosphatidylcholine hydrolysis, the effects of temperature switches upon DAG levels were assessed (Fig. 5). The results shown illustrate that activation of V-ABL (by a 39–32 °C temperature switch) resulted in a 1.89-fold increase in [3H]DAG levels in [3H]palmitate-labeled IC.DP cells, after a lag of 4 h. The reverse temperature switch stimulated a rapid decrease in [3H]DAG levels in these cells (Fig. 5A). Temperature shift experiments on the parent cell line resulted in no significant variation in [3H]DAG levels (Fig. 5A). In [3H]glycerol-labeled IC.DP cells, no significant changes in [3H]DAG were observed.

Involvement of Protein Kinase C in the Effects of the v-abl Tyrosine Kinase—DAG can activate the calcium, phospholipid-dependent protein kinase, PKC (Nishizuka, 1984). The

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**Fig. 4.** The effects of temperature switches upon the levels of water-soluble choline metabolites in IC2.9 and IC.DP cells. IC2.9 (closed symbols) and IC.DP (open symbols) cells were labeled to equilibrium with [3H]choline and maintained for 18 h at 39 or 32 °C before being washed free of IL-3 and incubated for 2 h prior to switching to 32 (squares) and 39 °C (circles), respectively, for the specified time intervals. Water-soluble [3H]choline metabolites: choline phosphate (A), choline (B), and glycerophosphocholine (C) were then analyzed by cation-exchange chromatography on Dowex-H* resin. The results were corrected to 100,000 disintegrations/min in the choline lipids and expressed as percent of the level in cells at 39 °C at time 0 h. The data points shown are mean of triplicate determinations from a single experiment representative of three. Standard deviation values did not exceed 10% of the mean value of any of the data points shown.

**Fig. 5.** The effects of temperature switches upon the levels of DAG in IC2.9 and IC.DP cells. IC2.9 (closed symbols) and IC.DP (open symbols) cells were labeled to equilibrium with either [3H]palmitate (A) or [3H]glycerol (B) and maintained for 18 h at 39 or 32 °C, washed free of IL-3, and incubated for 3 h prior to being switched to 32 (squares) and 39 °C (circles), respectively, for the specified time intervals. Cellular lipids were extracted and DAG separated by thin layer chromatography. DAG levels were calculated as a fraction of radioactivity in the total lipid and expressed as a percent of the level at time 0 h. Data shown are mean values from three (IC2.9) or five (IC.DP) experiments. S.E. values did not exceed 10% of the mean value of any data points shown.
role of this kinase in v-abl-stimulated IL-3-independent proliferation was therefore assessed.

Chronic treatment of cells with phorbol esters results in down-modulation of PKC (Rodriguez-Pena and Rozengurt, 1984). Long term (18 h) treatment of IC.DP cells with phorbol esters TPA and phorbol dibutyrate resulted in a reduction of total PKC activity to 2.74 ± 2.74% and 6.06 ± 3.95% (S.E. n = 3), respectively, relative to untreated control cells. The effect of PKC down-modulation upon [3H]thymidine incorporation in IC.DP cells cultured at 32 °C, in the absence of IL-3, is shown (Fig. 6A). Down-regulation of PKC resulted in an inhibition of thymidine incorporation. Similar effects were obtained with phorbol dibutyrate, but analogues of TPA which neither activate nor down-modulate PKC (β-phorbol, 4α-phorbol didecanoate, and 4-methoxy TPA) were found to have no effect upon [3H]thymidine incorporation (Fig. 6A).

We have also assessed the ability of the protein kinase C inhibitor, calphostin C, to inhibit the IL-3-independent proliferation of IC.DP cells cultured at 32 °C (this inhibitor is reported to have little effect upon the activity of members of the src tyrosine kinase family) (Tamaoki, 1991; Tamaoki and Nakano, 1990). There was a marked dose-dependent inhibition of [3H]thymidine incorporation under these conditions (see Fig. 6B). The dose at which half-maximal inhibition of [3H]thymidine incorporation occurred, 140 ± 10 nM (n = 3), was similar to that resulting in a 50% inhibition of protein kinase C activity in vitro (50 nm) (Tamaoki, 1991; Tamaoki and Nakano, 1990).

**The Role of Protein Kinase C in V-ABL-stimulated Na+/H+ Antiport Activation in IC.DP Cells**—Protein kinase C can activate the Na+/H⁺ antiport, resulting in an increase in pH (Bierman et al., 1987; Whetton et al., 1988b). In order to determine whether V-ABL activation can stimulate an increase in the activity of the Na+/H⁺ antiport, we have assessed the effects of temperature shifts upon the pH of the IC.2.9 and IC.DP cell lines (see Fig. 7 and Table I). Within 2 h of switching IC.DP cells from 39 to 32 °C, there was a small but detectable increase in pH, which then continued over the next 4 h (Fig. 7B). A reverse temperature shift from 32 to 39 °C was found to result in a rapid decrease in pH (Fig. 7A). Temperature shifts were found to have no such effects upon pH in the IC.2.9 cell line (see Table I).

In order to confirm that the increases in pH associated with activation of V-ABL were due to increased Na+/H⁺ antiport activity, we examined the recovery of pH, following NH₄Cl-induced acidification (Vallance et al., 1990). Despite a near identical degree of acidification in the pH of IC.DP cells maintained at 32 °C prior to the removal of ammonium ions recovered significantly faster than that of cells preincubated at 39 °C. Consistent with the data presented in Table I, these cells were also found to re-equilibrate to a higher steady state pH (Fig. 8B). In contrast, the recovery in pH observed under these conditions was found to be both 5-N-(methyl-N-isobutyl)amiloride (5-MNIA)-sensitive and dependent upon the presence of extracellular sodium ions (data not presented) and can therefore be attributed to the activity of the Na+/H⁺ antiport (Simchowitz and Cragoe, 1986).

Constitutive antiport activation in IC.DP cells maintained at 32 °C was further illustrated by their response to two agents that stimulate acute increases in pH, in the IC.2.9 cell line. Both IL-3 and TPA were found to stimulate a rapid increase in pH of IC.2.9 cells maintained at 39 and 32 °C (see Table I). However, while IL-3 and TPA stimulated corresponding increases in IC.DP cells at 39 °C, neither were found to stimulate any significant increase in the pH of IC.DP cells that had been maintained at 32 °C for 18 h (Table I). Furthermore, even after 6 h, addition of TPA stimulated no significant increase in pH in IC.DP cells maintained at 32 °C,
TABLE 1

Effect of temperature switch on resting pH, and responsiveness to IL-3 and TPA in IC2.9 and IC.DP cells

Following culture at 32 and 39 °C for 6 and 18 h, cells were incubated for a further 2 h in the absence of IL-3. Cells were loaded with BCECF prior to challenging with IL-3 (100 units/ml) or TPA (100 ng/ml) and pH, determined. Results are from a single experiment typical of three. Standard deviation values did not exceed 10% of the mean value of any of the data points shown.

| Cell line | Resting pH | ∆pH + IL-3 | ∆pH + TPA | Resting pH | ∆pH + IL-3 | ∆pH + TPA |
|-----------|------------|------------|-----------|------------|------------|-----------|
| After 6 h |            |            |           |            |            |           |
| IC2.9     | 6.98 ± 0.04| 0.11 ± 0.01| 0.14 ± 0.01| 7.00 ± 0.02| 0.14 ± 0.04| 0.14 ± 0.01|
| IC.DP     | 7.04 ± 0.02| 0.11 ± 0.02| 0.12 ± 0.04| 7.14 ± 0.02| 0.14 ± 0.04| 0.14 ± 0.01|
| After 18 h|            |            |           |            |            |           |
| IC2.9     | 7.02 ± 0.03| 0.14 ± 0.03| 0.15 ± 0.03| 7.04 ± 0.03| 0.14 ± 0.04| 0.14 ± 0.01|
| IC.DP     | 7.0 ± 0.01 | 0.11 ± 0.02| 0.12 ± 0.04| 7.25 ± 0.03| NSC        | NSC       |

* ND, not determined,  
NSC, no significant change.

FIG. 8. The effect of temperature shifts upon the rate at which IC2.9 and IC.DP cells recover from acute acidification. IC2.9 cells (A) and IC.DP cells (B) were cultured at 32 (squares) and 39 °C (triangles) for 18 h in the absence of IL-3, before being loaded with BCECF and acidified with ammonium chloride. The recovery of pH was then monitored and is shown in the inset diagrams. Results from such experiments were used to determine the relationship between pH, and its rate of change, as illustrated in the main diagrams. Data shown are representative of three such experiments. Standard deviation values did not exceed 10% of the mean value of any of the data points shown.

compared to an increase of 0.11 ± 0.02 in cells maintained at 39 °C (Table 1).

To investigate whether V-ABL exerts its effects upon the activity of the antagonist via PKC, the effect of PKC down-modulation was assessed. Cells were switched from 39 to 32 °C, and the increase in pH, over the next 6 h was determined. PKC down-modulated cells showed a rise in pH, which was 0.09 ± 0.04 (mean ± S.E., n = 3) pH units lower than that observed in control cells (which exhibited an increase in pH, of 0.125 over the same 6-h period). Furthermore, during the first 4 h after a 39–32 °C temperature switch, PKC-down-modulated cells showed no significant increase in pH, compared to the 0.05 pH unit increase observed in non-down-modulated control cells. It would therefore appear that V-ABL stimulates constitutive activation of the Na+/H+ antiport in IC.DP cells, and this is partially mediated by PKC.

DISCUSSION

Transfection of IL-3-dependent hemopoietic cells with Abelson murine leukemia virus leads to an abrogation of the requirement for growth factor via a non-autocrine mechanism (Cook et al., 1985; Pierce et al., 1985). Furthermore, temperature-sensitive mutants of the V-ABL tyrosine kinase have demonstrated a correlation between tyrosine kinase activity and IL-3-independent proliferation (Kipreos and Wang, 1988). The rapid and reversible effect of temperature upon the tyrosine kinase activity of these conditional mutants makes them ideal as model systems with which to study the biochemical role of v-abl in promoting autonomous survival and proliferation. Using the IC2.9 mast cell line transfected with the DP conditional mutant of v-abl (Kipreos et al., 1987; Kipreos and Wang, 1988), we have found that a temperature shift from 39 to 32 °C leads to the autophosphorylation of V-ABL, an event associated with the activation of its tyrosine kinase activity, and an increase in cellular phosphotyrosine levels. The rate at which a new equilibrium of V-ABL phosphorylation was achieved upon switching cells from the restrictive to the permissive temperature was comparable to that previously reported for the DP mutant (Kipreos and Wang, 1988). An analysis of the changes in second messengers in IC.DP cells upon temperature switch has revealed some of the cellular signaling events associated with the activation of this tyrosine kinase, and which may play a role in abrogating IL-3 dependence.

IL-3 is known to activate protein kinase C, probably by stimulating the generation of the second messenger DAG (Farrar et al., 1985; Whetton et al., 1986, 1988a, 1988b). The
main source of mitogen-stimulated increases in DAG was originally thought to be phospholipase C-mediated breakdown of PtdIns-4,5-P₂, which also generates IP₃. However, evidence indicates that IL-3 does not stimulate this pathway in IC.2.9 or other IL-3-dependent cell lines (Hamilton et al., 1989; Whetton et al., 1988a, 1988b).² Alternative sources of DAG have recently been characterized, e.g. phosphatidylcholine hydrolysis by phospholipases C and D (Billah and Anthes, 1990). There is some evidence to suggest that IL-3-stimulated DAG production occurs as a consequence of phosphatidylinositol hydrolysis (Duronio et al., 1989; Ruggiero et al., 1991). However in [³H]choline-labeled IC.2.9 cells, IL-3 had no effect on the levels of any of the water-soluble metabolites of phosphatidylinositol breakdown (over 60 min) suggesting that V-ABL is able to recruit alternative signaling pathways which can lead to the activation of protein kinase C.

Constitutive activation of v-abl in fibroblasts and in the hemopoietic 32D cell line indicated that DAG levels were elevated compared to those in the non-transfected parent cell lines (Fry et al., 1985; Ruggiero et al., 1991). In the 32D cell line, this rise in DAG was thought to result from phosphatidylcholine breakdown (Ruggiero et al., 1991). In IC.DP (but not IC.2.9) V-ABL activation from 39 to 32 °C resulted in an elevated level of choline phosphate in [³H]choline-labeled cells. A similar increase in DAG levels was observed in [³H]palmitate-labeled IC.DP cells (Fig. 5A), an effect which occurred after a lag of 4 h. As phosphatidylinositol contains relatively high proportions of palmitate fatty acid, it is likely that the main source of this DAG is phosphatidylinositol. The decrease in choline levels in IC.DP cells switched from 39 to 32 °C is probably due to recruitment of choline for the resynthesis of phosphatidylinositol.

All of the above changes in phospholipid metabolites occur with a lag phase of 4 h, and it is possible that V-ABL affects these pathways via the induction of protein synthesis. However, in the presence of the protein synthesis inhibitor cycloheximide (5 μM) the increase in [³H]DAG levels (after an 18 h switch from the restrictive to the permissive temperature) is 97 ± 15% (mean ± S.E., n = 3) of that observed in its absence. These results indicate that the changes in phospholipid metabolism are a direct effect of the v-abl tyrosine kinase and are not mediated through an induction of enzyme synthesis.

Preliminary data indicate that phosphatidic acid formation is not stimulated by a switch from the restrictive to the permissive temperature, suggesting that phospholipase D is not activated by the v-abl tyrosine kinase. Together these results suggest that activation of the v-abl tyrosine kinase stimulates phospholipase C-mediated breakdown of phosphatidylinositol, generating DAG, which may then activate protein kinase C.

When IC.DP cells were labeled with [³H]glycerol, in contrast to [³H]palmitate-labeled cells, no significant increase in DAG was observed as glycerol labeling techniques are less sensitive in detecting changes in DAG levels within a particular pool of lipid. Although there is an increase in the level of IP₃ in IC.DP cells when V-ABL is activated, further experiments will be required to establish whether Ptd-Ins₄,₅-P₂ hydrolysis contributes to any significant increase in the mass of DAG in IC.DP cells cultured at the permissive temperature. Preliminary experiments with [³H]inositol-labeled cells suggest that V-ABL activation does not increase the rate of inositol phospholipid turnover.³ Thus, elevated IP₃ levels may be a consequence of decreased rates of degradation of this second messenger in IC.DP cells cultured at 32 °C and not due to the increased breakdown of Ptd-Ins₄,₅-P₂.

Our data indicate that part of the mechanism whereby v-abl stimulates the abrogation of IL-3 dependence results from its ability to modulate phospholipid metabolism, thereby generating the second messenger DAG. It was anticipated that this would subsequently activate protein kinase C. Down-regulation of protein kinase C by chronic treatment with phorbol esters (Rodriguez-Pena and Rozengurt, 1984), or inhibition of its activity with the specific inhibitor calphostin C (Tamaoki, 1991; Tamaoki and Nakano, 1990), certainly decreases the rate of IL-3-independent thymidine incorporation observed in IC.DP cells cultured at the permissive temperature (see Fig. 6). Furthermore, we have used protein kinase C-mediated activation of the Na⁺/H⁺ antiport as a means of discerning whether some of the actions of the v-abl tyrosine kinase are mediated by PKC.

We have found that the proliferative stimulus of both V-ABL (activated by a 39–32 °C temperature switch) and IL-3 result in an increase in pH₇ of IC.DP cells. Parallel studies with the parent IC.2.9 cell line demonstrated that IL-3, but not temperature switches, stimulate increases in pH₇. Thus, the changes in pH observed in IC.DP cells do not arise from nonspecific effects of the temperature change employed.

Confirmation that the effects upon pH₇ of IC.DP cells are associated with increased Na⁺/H⁺ antiport activity comes from studies of the response of these cells to acute acidification. In bicarbonate-free medium, IC.DP cells cultured at 32 °C recover from acidification faster, and reach a higher steady-state pH₇, than those cultured at 39 °C, an effect dependent on the presence of extracellular sodium and inhibited by the amiloride analogue, 5-MNA (Simchowitz and Cragoe, 1988). Together, these experiments infer that v-abl can constitutively activate the Na⁺/H⁺ antiport. Down-modulation of protein kinase C partially inhibits the increase in pH₇ observed when IC.DP cells are switched from 39 to 32 °C. As IL-3 is known to activate the Na⁺/H⁺ antiport and stimulate cellular proliferation via a PKC-dependent mechanism (Whetton et al., 1988a, 1988b), it is likely that v-abl-mediated PKC activation is part of a cascade of events associated with the abrogation of growth factor dependence in IL-3-dependent cell lines. Thus, the effect of the v-abl tyrosine kinase on phospholipid breakdown is important in cell proliferation as a means of activating protein kinase C.

How do the effects of v-abl compare to those defined for other non-receptor tyrosine kinases? Most of the work in this area has employed the v-src oncogene, where the use of cell lines transfected with this oncogene has revealed remarkably similar effects to those reported here. The activation of v-src stimulates hydrolysis of phosphatidylinositol to generate the second messenger DAG (Diaz et al., 1990; Song et al., 1991), thereby activating protein kinase C (Durkin et al., 1990). Furthermore, both v-abl and v-src have been reported to stimulate the de novo synthesis of DAG (Chiarugi et al., 1987, 1989), although our data concerning phosphatidylinositol breakdown suggest that this is not the case in IC.DP cells in the short term.

Plainly, the substrates for V-SRC and V-ABL will not simply include enzymes which can influence phospholipid metabolism, although both can associate with phosphatidylinositol-3-kinase (Fukui and Hanafusa, 1989; Varticovski et al., 1991). We envisage that there will be major differences in the substrate specificity of these enzymes, which may govern the biological response elicited in transfected cells. To date, the only defined protein, other than phosphatidylinositol-3-kinase, shown to be phosphorylated as a consequence of V-

² C. A. Evans and A. D. Whetton, unpublished observations.
³ P. Musk and A. D. Whetton, unpublished observations.
ABL activation is C-RAF (Carroll et al., 1990), which is also phosphorylated by V-SRC and necessary for SRC function (Morrison et al., 1988; Qureshi et al., 1991). Using conditional mutants it will now be possible to identify some of the major proteins phosphorylated as a consequence of V-ABL activation and determine how these relate to the early signaling events associated with leukemic transformation by the abl oncogene.

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