Comparison of the levels of inositol metabolites in transformed haemopoietic cells and their normal counterparts

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We have compared the levels of inositol metabolites in three pairs of normal and transformed cells which have been matched with respect to their cell lineage, differentiation and proliferation status: (i) normal human myeloid blast cells and the human promyelocytic leukaemic cell line, HL60; (ii) human umbilical-cord T-helper cells and C8166 cells, a HTLV1-transformed T-helper cell line; and (iii) an interleukin 3-dependent long-term culture of murine pro-B-cells (BAF3) and BAF3 cells transformed by transfection with the bcr-*abl* oncogene. Complex patterns of inositol metabolites were present in each of the cell populations. Although there were a number of differences in the levels of certain inositol metabolites between individual cell populations in the paired groups, we did not observe any consistent difference in the levels of inositol metabolites between the proliferating normal and transformed cells. In particular, our data do not support the reported correlation between elevated glycerophosphoinositol (Gro*P*Ins) levels and transformation of cells by membrane and cytoplasmic oncogenes which has been reported by other workers. All the cells contained high concentrations of Ins(1,3,4,5,6)P_5 (between 12 and 55 μM) and InsP_6 (between 37 and 105 μM). The HTLV1-transformed T-helper cells had particularly high levels of total inositol phosphates (predominantly Gro*P*Ins, an unidentified inositol bisphosphate and InsP_6). The observations are discussed with reference to cell transformation and to the differentiation status of the paired populations.

INTRODUCTION

Malignancy is a breakdown in one or more of the cellular control systems which modulate the proliferation and specialization of cells in the body. Although many of the genetic events which can lead to malignancy have been described, the resulting biochemical changes in the target cells, which mediate the phenotype of malignancy, remain incompletely understood. The elucidation of these biochemical processes is important for two reasons. First, the identification of perturbations associated with tumorigenesis will reveal aspects of the control of normal cell growth and differentiation hitherto unknown. The most striking precedent for this has been the discovery of viral oncogenes, leading to the recognition of proto-oncogenes which are the normal cellular counterparts of viral oncogenes, which appear to play key roles in normal cell regulation. Secondly, it may become possible to identify metabolic targets for novel therapeutic intervention in the treatment of cancer.

Identification of these key biochemical events requires precise comparisons of transformed cells and normal cells. To be informative, studies must compare analyses of malignant cells, whose cell lineage, proliferation and differentiation status are well understood, with equivalent analyses of their normal cell counterparts. The haemopoietic system is uniquely suited to this kind of study, since it has been very extensively characterized. This has led to an understanding of the various blood-cell lineages and to the identification of cell populations at different stages of maturation within each lineage. Furthermore, the cellular origins of many of the leukaemias have been identified.

PtdIns(4,5)P_2 hydrolysis is one of the key intracellular signalling systems of the cell, many growth factors regulate inositol lipid 3-kinase, and cells also contain a number of highly phosphorylated inositol phosphates whose functions remain unclear (for reviews, see Downes and Macphee, 1990; Majerus et al., 1990). Thus perturbations in various aspects of the complex metabolism of inositol derivatives are potential candidates for biochemical changes that might contribute to malignancy. Indeed, there have been a number of reports that inositol metabolism is altered in malignant cells. For example, elevated levels of glycerophosphoinositol (Gro*P*Ins) have been reported in a number of transformed cells (Alonso et al., 1988; Alonso and Santos, 1990; Valitutti et al., 1991), elevated levels of Ins(1,4,5,6)P_4 have been observed after *src* transformation of Rat 1 fibroblasts (Johnson et al., 1989; Mattingly et al., 1991), activation of the inositol lipid 3-kinase has been reported during *abl* transformation (Varticovski et al., 1991), and the *trk* oncogene has been shown to associate with phospholipase C-γ1 (Ohmichi et al., 1991).

In a previous study, we compared the inositol-containing constituents of normal human myeloid blast cells purified from fetal liver with those of their transformed counterpart, the human promyelocytic cell line HL60 (Bunce et al., 1992). We have now extended these studies to include comparisons of two further matched pairs of haemopoietic cells and their transformed equivalents. The first of these comparisons is between non-transformed and transformed pro-B lymphocyte cell lines, and the second between normal and transformed T-helper cells (T_H). BAF3 cells are a long-term culture of murine B-cell progenitors whose proliferation in culture is driven by interleukin 3 (IL-3): the cells are not tumorigenic (Palacios and Steinmetz, 1985). A subclone of BAF3 cells has now been derived by transfection with a bcr-*abl* construct encoding the p210 bcr-*abl* tyrosine

Abbreviations used: FCS, fetal-calf serum; Ins(1,2-cyclic)*P*, inositol (1,2)-cyclic phosphate; Ins(?)P_n where n = number of phosphates and ? = position of phosphates on the inositol ring; the D numbering system is used [Biochem. J. (1989) **258**, 1–2]; Gro*P*Ins, *sn*-glycero-3-phospho-1-inositol; HTLV1, human T-cell lymphotrophic virus type 1; IL-3, interleukin 3; T_H, T-helper cells.

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kinase, whose constitutive expression renders the cells both growth-factor-independent *in vitro* and tumorigenic in mice. Primary T_H cells, identified by their expression of CD4 antigen in the absence of CD8 antigen, were isolated from cord blood samples and grown in culture under conditions described for the growth of unpurified T-cells (Cooper et al., 1989). These were compared with the human T-cell line C8166. This cell line was derived from cord blood cells transformed by the lymphotrophic HTLV1 virus which infects and transforms T_H cells (for review, see Gallo and Nervikar, 1989). The viral oncogene which transforms the cells is *tax*, which encodes a *trans*-activating factor (Tanaka et al., 1990).

In the present paper, we compare in detail the inositol-containing constituents of these three closely matched pairs of transformed and non-transformed cells in which the transforming events involve different nuclear and extra-nuclear oncogene products.

MATERIALS AND METHODS

Isolation and culture of cells

Cord blood CD4-positive T_H cells

Heparinized human cord blood samples (approx. 10 ml) were taken within 1 h of birth and diluted 1:2 in RPMI 1640 medium (Gibco, Paisley, U.K.) containing 2% (v/v) fetal-calf serum (FCS; Gibco) before centrifugation over Ficoll Hypaque (Pharmacia, Milton Keynes, U.K.). The resulting mononuclear-cell preparation was depleted of monocytes, B-cells and CD8-positive T-cells by coating these cells with mouse monoclonal antibodies, followed by binding of immunomagnetic beads coated with sheep antibodies to mouse Ig (Dynal, Merseyside, U.K.). Briefly, the mononuclear cells were washed twice in RPMI 1640 (containing 2% FCS) and resuspended at 2×10^7 cells/ml in the same medium containing saturating amounts of the antibodies 61D3 (recognizes monocytes and macrophages; Nunez et al., 1982), BU12 [anti-CD19 (B-cells); Binding Site, Birmingham, U.K.] and OKT8 [anti-CD8 (CD8-positive T-cells); American Type Culture Collection]. After 30 min at room temperature the cells were washed twice, magnetic beads were added directly to the cell pellet at a ratio of 5 beads per cell and the mixture was incubated on ice for 3 min. The cell suspension was diluted to 5 ml, and antibody-coated cells which had bound magnetic beads were removed with a magnet (Dynal).

The remaining negatively selected cell population was cultured in 24-well tissue-culture plates (Nunc, Paisley, U.K.) at an initial density of 1×10^6 cells/ml in RPMI 1640 containing penicillin (100 units/ml), streptomycin (100 μg/ml) and 2 μCi/ml *myo*-[^{3}H]inositol (Amersham International, Amersham, Bucks., U.K.) and supplemented with 10% FCS and 2 μg/ml phytohaemagglutinin (Sigma, Poole, Dorset, U.K.). After 72 h the cells were harvested and washed once before being replaced in culture in identical labelled medium but containing 200 μg/ml human recombinant interleukin 2 (Eurocetus, Harefield, U.K.) in place of phytohaemagglutinin. The cells were cultured for a further 6 days, during which time fresh medium was given on at least two occasions. Before making extracts of the cells, cells expressing CD4 in the absence of CD8 were again enriched by negative selection. This procedure used the same antibody cocktail as described above, but unwanted cells were removed by indirect rosetting by using sheep red cells coated with antibodies to mouse Ig as described previously (Bunce et al., 1990, 1992). The enrichment of CD4-positive cells was monitored, by enumerating their numbers using monoclonal anti-CD4 (OKT4; American Type Culture Collection) and the indirect rosette assay. The mean of values from four experiments was 90%.

Myeloid blast cells

The procedures for negatively selecting normal human myeloid blast cells from fetal liver, their enrichment by elutriation and their labelling with [^{3}H]inositol have been described previously (Bunce et al., 1992).

HL60 promyelocytic cells

Cultures of HL60 cells were maintained in RPMI 1640 medium containing antibiotics as above and supplemented with a commercial serum replacement which contains insulin, transferrin, selenium dioxide, linoleic acid and BSA (ITS+; Universal Biologicals, Merseyside, U.K.). The inositol concentration in this medium was therefore 35 mg/l, and thus differs from that used in our previous study (French et al., 1991) in which the medium contained 1 mg/l inositol.

C8166 T-cell line

The T-helper cell line C8166 (Salahuddin et al., 1983) was maintained in RPMI 1640 medium containing antibiotics and supplemented with 10% FCS.

The BAF3 B-cell line and its transformants

BAF3 transfectants were generated by electroporation of cells in the presence of 10 μg of plasmid DNA encoding bcr-*abl* p210 driven from the expression vector $_p$M5 (Laker et al., 1987). A 'Bio-Rad' gene pulser apparatus was used to deliver 625 $V \cdot cm^{-1}$ from a 500 μF capacitor to 5×10^5 BAF3 cells which had been previously washed and resuspended in RPMI 1640 plus 100 μg/ml salmon sperm DNA. Transfectants were selected in the presence of 2 mg/ml G418. Bcr-*abl* p210 expression was confirmed by immune-complex kinase assay (results not shown). BAF3 cultures and cultures of their bcr-*abl*-transformed subclone were maintained in the same media as the C8166 cells, except that murine recombinant IL-3 was added to BAF3 cultures. This was supplied by the addition of 1% (v/v) conditioned and filtered culture supernatant from NIH-3T3 cells transfected with the murine IL-3 gene (Karasuyama and Melchers, 1988).

Extraction of inositol metabolites and separation of soluble metabolites by h.p.l.c.

All of the above cultures were labelled by addition of 2 μCi/ml [^{3}H]inositol into the medium. Cultures of HL60, BAF3 and bcr-*abl*-transformed BAF3 cells were labelled for at least 6 days before being extracted, whereas C8166 cells were labelled for 14 days before extraction; these different labelling periods reflect the time taken for all of the inositol phosphates to reach equilibrium in each of the cell types. That equilibrium was reached was certain, since each of the cultures was labelled over several cell doubling times.

Cells labelled to equilibrium with *myo*-[^{3}H]inositol were extracted and the water-soluble inositol metabolites separated by h.p.l.c. as previously described (French et al., 1991), except that a 25 cm Partisphere 5 SAX column with an AX guard cartridge (Laserchrom Analytical, Gravesend, U.K.) was used. Radioactivity was quantified either by collecting fractions followed by liquid-scintillation counting using Flow Scint IV scintillant (Canberra Packard, Pangbourne, U.K.) or by using a

Radiomatic on-line radiochemical detector (update time 30 s), with Flow Scint IV (5:1 scintillant:mobile phase). Intracellular concentrations of the various inositol metabolites detected were calculated from the radioactivity in each h.p.l.c. peak, the total cell volume and the specific radioactivity of the [^{3}H]inositol in the culture medium as previously described (French et al., 1991). The C8166 cells grew in large clumps. In order to make accurate cell counts and to determine cell size, single-cell suspensions of C8166 cells were prepared by passing cultured cells through a 19-gauge needle. C8166 cells prepared in this way were the most irregular in shape of all of the cells studied. Therefore we estimate that the resulting errors in determining the cell size could introduce variations into the calculations of intracellular inositol metabolite concentrations of up to 20%. The concentration of inositol in the culture medium was measured by a chemiluminescence assay (Gudermann and Cooper, 1986).

Deacylation of lipid extracts and separation of resulting Gro*P*Ins esters by h.p.l.c.

Lipid extracts were deacylated essentially as described by Clarke and Dawson (1981), except that 1 mg of a brain lipid extract (B-1502; Sigma) was added to each lipid extract as an unlabelled carrier and that 10 mM EDTA was present during the deacylation step. The resulting Gro*P*Ins esters were separated by h.p.l.c. on a 25 cm Partisphere 5 SAX column with an AX guard column by using the following gradient with water in pump A and initially 0.1 M ammonium phosphate (pH 3.8) in pump B, switching to 1 M ammonium phosphate (pH 3.8) at 65.1 min: 0 min 0% B, 5 min 0% B, 35 min 30% B, 35.1 min 100% B, 65 min 100% B, 65.1 min 30% B, 95 min 30% B, 95.1 min 70% B, 110 min 70% B, 110.1 min 0% B. The column was equilibrated with water for at least 30 min before injection of each sample. Radioactivity was quantified with a Radiomatic on-line radiochemical detector with Flow Scint IV scintillant (4:1 scintillant:mobile phase) and an update time of 20 s.

RESULTS

Concentrations of inositol, inositol lipids and water-soluble inositol metabolites in cells

Table 1 records the concentrations, averaged throughout the measured cell volumes, of the above components in the six cell populations under study. Since the lipids will be localized to membranes and the water-soluble metabolites to one or more of the aqueous compartments of the cell, the real local concentrations of each component will be larger, by unknown factors, than the values recorded.

Inositol

The free inositol content of each cell type will be a consequence of some combination of the medium inositol content and of the relative rates of inositol entry and utilization. In the present experiments, the pro-B and T-cells were grown in a culture medium with a high concentration of inositol (0.5 mM). The intracellular inositol levels of the pro-B cell lines, at 0.6–0.8 mM, were a little higher than in their growth medium, whereas the T-cells accumulated substantial amounts. The intracellular inositol concentration was particularly high in the umbilical-cord T-cells, suggesting that these cells may have a particularly active transport system for accumulation of inositol. The normal myeloid blast cells were supplied with less inositol (approx. 27 μM), but also accumulated substantial quantities: the data for normal myeloid blast cells in Table 1 come from 14 independent analyses, including the four sets of data previously reported in Table 2 of Bunce et al. (1992). We have grown HL60 cells successfully both at 5.5 μM inositol (French et al., 1991) and at 190 μM inositol (Table 1). The intracellular inositol concentration was maintained at 7 and 15 times the extracellular concentration respectively.

Inositol lipids

Except for the C8166 HTLV1-transformed T_H-cells, all of the cells contained similar amounts of PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 (Table 1). It is not clear why the C8166 cells contain 2–3 times as much of the three inositol lipids as their untransformed T_H-cell counterparts. The PtdIns content of HL60 cells reported here is about double that which we reported previously in cells that had been grown with a more limited inositol supply (French et al., 1991), but the levels of PtdIns4*P* and PtdIns(4,5)P_2 were less influenced by the inositol supply. The more recently discovered 3-phosphorylated lipids are present at low levels within cells and are therefore difficult to detect when cells are labelled with [^{3}H]inositol. Consequently we have not attempted to make a detailed study of these lipids. A peak corresponding to deacylated PtdIns3*P* was occasionally observed in extracts from BAF3 cells and P210 cells. Its concentration was of the order of 0.04 μM, and did not appear to differ between the normal and transformed populations.

Total water-soluble inositol metabolites

Figure 1 shows typical anion-exchange h.p.l.c. separations of the [^{3}H]inositol-labelled metabolites present in aqueous extracts of equilibrium-labelled BAF3 cells and their bcr-*abl*-transformed counterparts. These traces show that the relative levels of the various inositol metabolites in these two types of cells were essentially identical.

The myeloid cells and pro-B cells all contained water-soluble inositol metabolites at total concentrations between about 150 μM and 280 μM, whereas the T-cells, especially the HTLV1-transformed C8166 cells, contained considerably higher concentrations of these metabolites (360 μM) (Table 1).

Gro*P*Ins

This is eluted within an asymmetric peak (peak B in Figure 1 and Table 1) between the unretarded inositol peak and the multiple inositol monophosphate isomers (Figure 1): at least in HL60 cells, > 90% of the material in this peak is Gro*P*Ins (French et al., 1991). This peak is also likely to have been homogeneous Gro*P*Ins in the other cell types, since it constantly co-eluted with authentic [^{14}C]Gro*P*Ins and showed an identical asymmetry. Some differences in Gro*P*Ins levels were observed between the different cell pairings. The myeloid and pro-B cells all had Gro*P*Ins contents of 15–27 μM, whereas the T_H-cells contained about 4 times as much (60–100 μM). It may be significant that the C8166 line contained the highest quantity both of Gro*P*Ins and of PtdIns, its likely precursor.

As outlined in the Introduction, previous studies have suggested that increased cellular levels of Gro*P*Ins are characteristic of all cells that have been transformed by oncogenes which are located either at the cell membrane or in the cytosol. Two of the cells analysed in the present study fall into this category, namely the bcr-*abl*-transformed pro-B-cell line and

Table 1 Concentrations of inositol, water-soluble inositol metabolites and inositol lipids in normal and transformed cells

[^{3}H]Inositol and water-soluble inositol metabolites extracted from growing cells which had been labelled to equilibrium were separated by anion-exchange h.p.l.c. The various inositol metabolites detected are listed in the order in which they were eluted from the column. Where identities have been given, these have been confirmed either by co-migration of standards or as described in previous studies (French et al., 1991; Bunce et al., 1992). Lipid extracts of the same [^{3}H]inositol-labelled cells were deacylated and the resulting Gro*P*Ins esters separated by h.p.l.c. as described in the Materials and methods section. The data are shown as concentrations (μM averaged throughout the cell volume) calculated from the specific radioactivity of the [^{3}H]inositol in the medium and the measured sizes of the cells. The concentration of inositol in the culture medium used for each cell type is given. The data are means ± S.E.M. of 3 determinations for HL60 cells, 4 determinations for both BAF3 cells and their bcr-*abl*-transformed subclone (P210), and 5 and 4 determinations for normal T_H-cells and C8166 cells respectively. Data for the myeloid blast cells have been published previously (Bunce et al., 1992), but the data shown here represent the mean of a larger series of experiments ($n = 14$ rather than 4). The data shown here for HL60 cells differ from those in a previous report, since the cells were grown in 35 mg/l inositol rather than 1 mg/l. Abbreviation: N.S., not separated from preceding peak.

		Cell cultures					
Peak	Inositol metabolites	Normal myeloid blasts	HL60	BAF3	P210	T_H	C8166
A	Inositol	3277 ± 679	2888 ± 481	625 ± 166	758 ± 141	19800 ± 3600	2730 ± 250
	Inositol phosphates						
B	Gro*P*Ins	26.8 ± 4.1	17.1 ± 1.7	15.7 ± 2.8	17.0 ± 5.1	61.9 ± 11.9	100.5 ± 20.8
C	Ins1/3*P*	20.8 ± 4.1	21.8 ± 1.7	16.9 ± 6.2	23.2 ± 6.5	50.3 ± 5.2	48.4 ± 10.0
D	Ins2*P*	9.6 ± 2.6	3.8 ± 1.8	37.3 ± 10.5	43.0 ± 7.9	41.3 ± 6.2	29.0 ± 4.0
E	Ins4/5/6*P*	1.1 ± 0.3	1.5 ± 0.3	0.4 ± 0.1	0.9 ± 0.3	1.7 ± 0.6	5.8 ± 1.0
F	Ins(1,3)P_2	< 0.2	2.1 ± 0.1	0.8 ± 0.4	1.2 ± 0.3	0.9	1.1 ± 0.1
G	Ins(1,4)P_2	15.7 ± 2.4	3.7 ± 0.5	0.9 ± 0.3	1.3 ± 0.3	3.0 ± 1.9	22.6 ± 2.4
H	Ins(?)P_2	< 0.2	10.9 ± 1.2	13.9 ± 5.6	12.4 ± 3.2	12.5 ± 3.3	42.4 ± 4.8
I	Ins(?)P_2	N.S.	N.S.	1.2 ± 0.0	1.6 ± 0.2	2.4 ± 1.4	1.4 ± 0.0
J	?	< 0.2	0.4 ± 0.1	< 0.6	< 0.6	< 0.9	< 1.0
K	Ins(?)P_3	< 0.2	13.1 ± 2.1	3.4 ± 2.7	5.6 ± 2.9	4.8 ± 1.5	2.2 ± 0.3
L	Ins(?)P_3	< 0.2	0.9 ± 0.2	0.6 ± 0.2	0.6 ± 0.3	< 0.9	< 1.0
M	Ins(1,4,5/?)P_3	2.9 ± 0.7	1.8 ± 0.1	1.0 ± 0.3	1.4 ± 0.3	2.4 ± 1.4	5.7 ± 1.35
N	InsP_4	4.4 ± 0.6	3.3 ± 0.5	5.9 ± 1.7	6.3 ± 1.2	2.9 ± 1.9	2.1 ± 0.5
O	Ins(1,3,4,5,6)P_5	48.1 ± 5.2	21.8 ± 3.0	51.5 ± 9.8	54.6 ± 10.5	49.4 ± 4.5	12.2 ± 3.1
P	Ins(?)P_5	< 0.2	2.1 ± 0.4	2.3 ± 0.6	1.8 ± 0.1	< 0.9	< 1.0
Q	Ins(?)P_5	< 0.2	2.2 ± 0.5	2.1	1.9 ± 0.2	< 0.9	2.9 ± 0.7
R	InsP_6	37.3 ± 3.8	39.5 ± 2.5	89.8 ± 20.1	105.1 ± 22.0	44.1 ± 3.7	88.2 ± 20.1
	Total Ins*P*	167	146	244	278	278	365
	Inositol lipids						
	PtdIns	1404 ± 105	897 ± 125	2147 ± 418	1600 ± 423	1960 ± 196	3617 ± 663
	PtdIns(4)*P*	58 ± 12	31 ± 3	63 ± 19	42 ± 13	131 ± 22	331 ± 72
	PtdIns(4,5)P_2	34 ± 7	32 ± 18	47 ± 10	43 ± 16	65 ± 21	159 ± 43
	Cell volume (μm^3)...	855	959	573	875	459	694
	[Medium inositol] (μM)...	27	193	514	514	514	514

HL60 cells, which express an activated N-*ras* gene (Murray et al., 1983). By contrast, the transforming element in the HTLV1-transformed T-cells is located in the nucleus. We therefore expected to find elevated Gro*P*Ins levels in the former two lines. However, as is shown in Table 1, the levels of Gro*P*Ins were similar in bcr-*abl*-transformed BAF3 cells and in the parental BAF3 cells, and also in HL60 cells and normal myeloid blast cells. Thus we observed no correlation between cell transformation by these particular membrane and cytoplasmic oncogenes and the cellular levels of Gro*P*Ins. By contrast, there was a difference in the Gro*P*Ins contents of the T_H-cells transformed by the nuclear oncogene *tax* and their normal counterpart, the Gro*P*Ins level being slightly higher in the transformed T_H-cells (Table 1).

Inositol monophosphates

There were similar total concentrations of inositol monophosphates within each of the cell pairings, but these concentrations varied among the cell types: the myeloid cells, pro-B-cells and T-cells contained Ins*P*s at total concentrations of approx. 30, 60 and 90 μM respectively. There were also differences in the relative levels of the various inositol monophosphate isomers: Ins1*P* and/or Ins3*P* (peak C) predominated in the myeloid and T-cells, whereas Ins2*P* (peak D) was most abundant in the pro-B-cells.

Inositol bisphosphates

All of the cells except the C8166 line contained inositol bisphosphates at total concentrations in the range 15–20 μM: Ins(1,4)P_2 predominated in myeloid blasts, but the predominant InsP_2 in the other cells was a partially characterized peak which in HL60 cells contained Ins(1,2)P_2 or its enantiomer Ins(2,3)P_2 and which was eluted later from the Partisil 5 SAX column (peak H). This peak was eluted in the position expected for Ins(3,4)P_2; however, this compound was not detected in extracts of unstimulated HL60 cells (P. J. French, unpublished work). The other InsP_2 (peak I) did not co-elute with Ins(4,5)P_2 and remains unidentified. The C8166 cells contained about three times as much total InsP_2 as the other cells, comprising mainly the unidentified isomer in peak H and Ins(1,4)P_2 at a ratio of about 2:1.

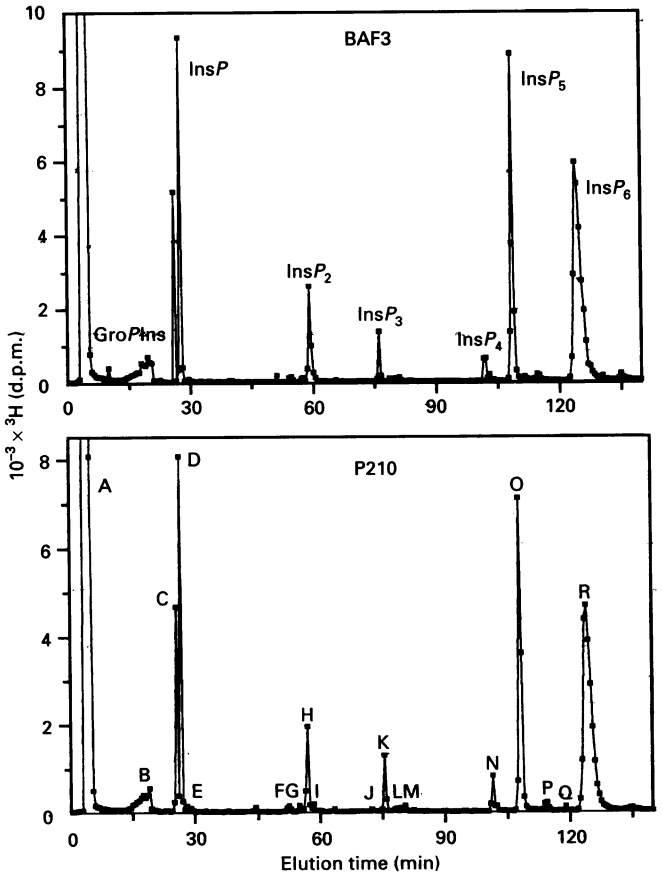

Figure 1 Identification of soluble inositol metabolites in growing factor-dependent pro-B-cells (BAF3) and bcr-*abl*-transformed pro-B-cells (P210)

The culture and labelling of cells with [^{3}H]inositol and their subsequent extraction are described in the Materials and methods section. This Figure shows typical anion-exchange h.p.l.c. obtained from similar numbers of BAF3 cells and their bcr-*abl*-transformed subclone (P210). Peaks corresponding to inositol and Gro*P*Ins and regions corresponding to increasingly phosphorylated forms of inositol are highlighted. In addition, each peak is identified by a letter which corresponds to the peaks listed in Table 1.

Inositol trisphosphates

The low levels of most InsP_3 isomers were difficult to assay accurately in these studies in which the cells were mainly grown in media containing inositol labelled to only relatively low specific radioactivity. As reported previously (French et al., 1991), the main InsP_3 in HL60 cells (peak K) remains structurally unassigned, and a peak eluted in the same position was a feature of the T_H-cells. The other major InsP_3, which co-eluted with Ins(1,4,5)P_3, ranged in concentration between about 2 and 6 μM. How much of the material in peak M is authentic Ins(1,4,5)P_3 is not certain, since in HL60 cells this peak also contains Ins(3,4,5)P_3 and/or its enantiomer. Because cell numbers were limiting, this peak was the only InsP_3 detectable in extracts of normal myeloid blast cells.

Inositol tetrakisphosphates

The levels of inositol tetrakisphosphate(s) in all of the cells were similar, at about 2–6 μM. In HL60 cells, this peak contains multiple InsP_4 isomers (Pittet et al., 1989), and this is likely also to be true for the other cells.

Inositol pentakisphosphates and hexakisphosphate

All of the cells contained substantial quantities of InsP_5 and InsP_6. These were present at combined concentrations of about 65–160 μM. InsP_6 was particularly abundant, relative to InsP_5, in the C8166-transformed T_H-cell line, which contained 88 μM InsP_6 and only 12 μM InsP_5.

DISCUSSION

In this study we have compared the levels of the inositol phosphates and the inositol-containing lipids in three matched pairs of normal and transformed cells to determine whether there are any changes in the metabolism of inositol which contribute to the malignant phenotype. In each pairing the oncogenes believed to be responsible for the transformation of the cells are known. The paired combinations were; (i) normal human myeloid blasts cells and HL60 cells (containing an N-*ras* mutation and an elevated c-*myc*), (ii) a murine IL-3 dependent pro-B-cell line (BAF3) and the same cell line transfected with the bcr-*abl* oncogene, and (iii) normal human T_H-cells and HTLV1-transformed T_H-cells (*tax* oncogene).

Alonso et al. (1988) and Alonso and Santos (1990) have reported elevated Gro*P*Ins levels, which appear to be a consequence of an increase in phospholipase A_2 activity, in NIH-3T3 fibroblasts transfected with oncogenes which exert their action either at the cell membrane (*ras*, *src*, *met* and *trk*) or in the cytoplasm (*mos* and *raf*) and in Rat 1 fibroblasts transfected with *ras*. Elevated Gro*P*Ins levels were not observed in Rat 1 fibroblasts transfected with the nuclear oncogenes *fos* and *myc*. Furthermore, the level of Gro*P*Ins in a *ras*-transfected NIH-3T3 cell line which had spontaneously reverted to the untransformed phenotype was similar to that observed in the parental untransformed cells. Alonso and co-workers have interpreted these findings to suggest that an elevated Gro*P*Ins level is a universal biochemical marker of malignancy when cells are transformed with either cytoplasmic or membrane oncogenes, but not nuclear oncogenes. Valitutti et al. (1991) have also reported elevated levels of Gro*P*Ins in *ras*-transfected FRTL5 cells, a rat thyrocyte cell line. However, Europe-Finner et al. (1988) did not observe an increase in Gro*P*Ins after *ras* transfection of *Dictyostelium discoideum*.

Two of the transformed cell populations used in the present study express membrane-associated or cytosolic oncogenes: HL60 cells, which have an activated N-*ras* (Murray et al., 1983) and the bcr-*abl*-transformed pro-B-cells. In neither of these transformed populations did we observe any significant change in the levels of Gro*P*Ins when compared with the normal counterpart. Thus it seems that an elevated Gro*P*Ins level is not a universal marker of transformation by either membrane or cytoplasmic oncogenes. In contrast, there was some indication that Gro*P*Ins levels may be somewhat elevated in T_H-cells transformed by the HTLV1-derived nuclear oncogene *tax*. The reason for this discrepancy between our findings and those reported previously is unclear. One possibility is that the elevated Gro*P*Ins levels reported previously were non-specific effects of the transfection process. Valitutti et al. (1991) had difficulty culturing their *ras*-transfected FRTL5 cells, and Ross et al. (1991) have reported that NIH-3T3 cells transfected with a vector control grew to a lower cell density that did untransfected NIH-3T3 cells.

One of the consequences of transformation of haemopoietic cells such as the BAF3 pro-B cells is to make their growth and survival independent of added IL-3. We therefore considered the possibility that transformation might convert the generation of Gro*P*Ins, presumably initiated by phospholipase A_2 activation,

from a dependence on sustained activation by growth factors to an oncogene-driven and constitutive mode. This interpretation accords with observations on *myc*, *ras* and other proto-oncogenes altered as a result of chromosomal translocation or point mutation and whose impact relies more on the constitutive sustained activation rather than levels of the product itself (Klein and Klein, 1985; Bizub et al., 1987; Freytag, 1988). To test this possibility, BAF3 cells were grown and labelled in the presence of IL-3, which was then removed for 5 h, the longest period compatible with full cell survival, before returning the cells to medium containing IL-3. During this treatment, the Gro*P*Ins level remained stable (results not shown). Thus Gro*P*Ins generation does not seem to be involved in the IL-3-driven proliferation of BAF3 cells.

Inositol 1,2-cyclic phosphate [Ins(1,2-cyclic)*P*] is an inositol metabolite which has not been assayed in this study, but whose concentration and metabolism have also been linked to cell transformation. Ins(1,2-cyclic)*P* is present at high levels (70 μM) in Morris (7777) hepatomas (Graham et al., 1987), an observation which has been linked to a decrease in the activity of Ins(1,2-cyclic)*P* 2-phosphohydrolase ('cyclic hydrolase') in these cells (Ross and Majerus, 1991). A decreased cyclic hydrolase activity was also observed in *src*-transformed Balb-3T3 fibroblasts. Ross et al. (1991) increased the cyclic hydrolase activity in NIH-3T3 cells by transfection with cloned cyclic hydrolase. This resulted in a decline in the level of Ins(1,2-cyclic)*P* in the cells and appeared to cause a progressive increase in their sensitivity to contact inhibition. Thus changes in the levels of Ins(1,2-cyclic)*P* may be involved in the loss of contact inhibition which is a phenotypic characteristic of transformed fibroblasts.

Ross and Majerus (1991) observed that Gro*P*Ins was also metabolized by cyclic hydrolase *in vitro*. Extracts from normal tissues had a higher activity towards Ins(1,2-cyclic)*P* than Gro*P*Ins, but they reported that there was a change in the substrate specificity of cyclic hydrolase, towards a preference for Gro*P*Ins, as cells adopted a more transformed phenotype: no mechanism was offered for this change in substrate specificity. These results could suggest that the elevated Gro*P*Ins levels reported by Alonso and colleagues (Alonso and Santos, 1988; Alonso et al., 1990) and by Valitutti et al. (1991) contributed indirectly to the loss of contact inhibition in the transformed cells by inhibiting cyclic hydrolase and thus raising Ins(1,2-cyclic)*P* levels. Since the cells investigated in the study reported here do not normally undergo contact inhibition, any such mechanism would not be relevant to their growth regulation.

Although we observed no significant differences in the levels of Gro*P*Ins within the pairings of normal and transformed cells, there were differences between the cell pairs. In particular, the T_H-cells had very much higher concentrations of Gro*P*Ins, between 60 and 100 μM. Possible explanations for this could involve differences between cell lineages or between cells at different stages of differentiation. The T-cells are a mature cell population, whereas the myeloid cells and pro-B-cells are both immature, and we have previously shown that Gro*P*Ins levels increase during myeloid differentiation (French et al., 1991; Bunce et al., 1992).

The HTLV1-transformed T_H-cells (C8166) also contained higher levels of some other aqueous inositol metabolites and of inositol-containing phospholipids than the normal T_H-cells and the myeloid and pro-B-cells. C8166 cells grow in clumps in culture, and for most experiments they were dispersed by passage through a fine needle before extraction; identical profiles were obtained when the cells were directly extracted without such dispersion. The reason for these differences therefore remains unknown, but they were only observed in the T_H-cell comparison, so are unlikely to be a feature of transformation itself. The C8166 cells contained high levels of Ins(1,4,5)P_3, Ins(1,4)P_2 and Ins4*P* (and/or Ins6*P*), metabolites which are produced upon agonist-induced breakdown of PtdIns(4,5)P_2. The high levels of the major InsP_2 (peak H) in C8166 cells, is of interest, because it may point to an unusual pattern of metabolism of the inositol polyphosphates in these cells. Clarification of whether the high levels of inositol metabolites in the C8166 cells represent a general consequence of *tax* transformation would require the analysis of several *tax*-transformed clones derived from separate donors.

In conclusion, in these studies of the effects of cell transformation on cellular levels of inositol and its metabolites we have used matched pairings of untransformed and transformed cells and have found no consistent features that characterize transformation. The inositol-containing compounds studied have only been formally identified in HL60 cells, but it is unlikely that the constituents of co-eluting peaks differ greatly in extracts of the other cells.

We do not know why these results contrast so strikingly with the apparently clear association between Gro*P*Ins accumulation and transformation by membrane and cytoplasmic oncogenes that has been reported previously by others and which provoked these studies. Whatever the reason for this discrepancy, our new information must cast serious doubt on the validity of any model of cell transformation, by these oncogenes, that seeks to rely in any essential way on control of the pathways that form or degrade Gro*P*Ins.

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REFERENCES

Alonso, T. and Santos, E. (1990) Biochem. Biophys. Res. Commun. **171**, 14–19
Alonso, T., Morgan, R. O., Marivzon, J. C., Zarbl, H. and Santos, E. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 4271–4275
Bizub, D., Heimer, E. P., Felix, A., Chizzonite, R., Wood, A. and Skalka, A. M. (1987) Oncogene **1**, 131–142
Bunce, C. M., Patton, W. N., Pound, J. D., Lord, J. M. and Brown, G. (1990) Leuk. Res. **14**, 1007–1018
Bunce, C. M., French, P. J., Patton, W. N., Scott, S. A., Michell, R. H. and Brown, G. (1992) Proc. R. Soc. London B **247**, 27–33
Clarke, N. G. and Dawson, R. M. C. (1981) Biochem. J. **195**, 301–306
Cooper, H. L., McDuffie, E. and Braverman, R. (1989) J. Immunol. **143**, 956–963
Downes, C. P. and Macphee, C. H. (1990) Eur. J. Biochem. **193**, 1–18
Europe-Finner, G. N., Ludérus, M. E. E., Small, N. V., Van Driel, R., Reymond, C. D., Firtel, R. A. and Newell, P. C. (1988) J. Cell. Sci. **89**, 13–20
French, P. J., Bunce, C. M., Stephens, L. R., Lord, J. M., McConnell, F. M., Brown, G., Creba, J. A. and Michell, R. H. (1991) Proc. R. Soc. London B **245**, 193–201
Freytag, S. O. (1988) Mol. Cell. Biol. **8**, 131–142
Gallo, R. C. and Nervikar, L. A. (1989) Ann. N.Y. Acad. Sci. **567**, 82–94
Graham, R. A., Meyer, R. A., Szwergold, B. S. and Brown, R. R. (1987) J. Biol. Chem. **262**, 35–37
Gudermann, T. W. and Cooper, T. G. (1986) Anal. Biochem. **158**, 59–63
Johnson, R. M., Wasilenko, W. J., Mattingly, R. R., Weber, M. J. and Garrison, J. C. (1989) Science **246**, 121–124
Karasuyama, H. and Melchers, F. (1988) Eur. J. Immunol. **18**, 97–104
Klein, G. and Klein, E. (1985) Nature (London) **315**, 190–195
Laker, C., Stocking, C., Bergholz, U., Hess, N., De Lamarter, J. F. and Ostertag, W. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 8458–8462
Majerus, P. W., Ross, T. S., Cunningham, T. W., Caldwell, K. K., Jefferson, A. B. and Bansal, V. S. (1990) Cell **63**, 459–465
Mattingly, R. R., Stephens, L. R., Irvine, R. F. and Garrison, J. C. (1991) J. Biol. Chem. **266**, 15144–15153
Murray, M. J., Cunningham, J. M., Parada, L. F., Dautry, F., Lebowitz, P. and Weinberg, R. A. (1983) Cell **33**, 749–757
Nunez, G., Ugolini, V., Capra, J. D. and Stastny, P. (1982) Scand. J. Immunol. **16**, 515–523

Ohmichi, M., Decker, S. J., Pang, L. and Saltiel, A. R. (1991) J. Biol. Chem. **266**, 14858–14861
Palacios, R. and Steinmetz, M. (1985) Cell **41**, 727–734
Pittet, D., Schlegel, W., Lew, D. P., Monod, A. and Mayr, G. W. (1989) J. Biol. Chem. **264**, 18489–18493
Ross, T. S. and Majerus, P. W. (1991) J. Biol. Chem. **266**, 851–856
Ross, T. S., Whiteley, B., Graham, R. A. and Majerus, P. W. (1991) J. Biol. Chem. **266**, 9086–9092

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Salahuddin, S. Z., Markham, P. D., Wong-Staal, F., Franchini, G., Kalyanaraman, V. S. and Gallo, R. C. (1983) Virology **129**, 51–64
Tanaka, A., Takahashi, C., Yamoaka, S., Nosaka, T., Maki, M. and Hatanaka, M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1071–1075
Valitutti, S., Cucchi, P., Coletta, G., Di Filippo, C. and Corda, D. (1991) Cell. Signalling **3**, 321–332
Varticovski, L., Daley, G. Q., Jackson, P., Baltimore, D. and Cantley, L. C. (1991) Mol. Cell. Biol. **11**, 1107–1113