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Mitogenic signal transduction in normal and transformed 32D hematopoietic cells

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We studied mitogenic signal transduction in normal and oncogene-transformed 32D cells, a murine hematopoietic cell line that is normally dependent on interleukin-3 (IL3) for proliferation and survival The formation of second messengers was measured in normal cells stimulated with IL3, and in cells transfected with foreign growth factor receptor genes and stimulated with appropriate growth factors We also measured the steady-state level of second messengers in 32D cells transformed by *erb*B, *abl*, and *src* oncogenes which abrogate growth factor requirement We found that IL3 stimulated the formation of diacylglycerol independently of inositol lipid turnover, but concomitantly with increased turnover of phosphatidylcholine Epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) stimulated the 'classical' turnover of inositol lipids with formation of diacylglycerol and calcium-mobilizing inositol phosphates Colony stimulating factor-1 trigged inositol lipid turnover, although to a much lower extent than EGF and PDGF Transformed cells showed elevated levels of diacylglycerol together with increased turnover of phosphoinositides and phosphatidylcholine Taken together these results indicate that different growth factors and oncoproteins associate with multiple signalling pathways in 32D cells

Hematopoietic cell, Growth factor, Oncogene, Inositol lipid, Diacylglycerol, Phosphatidylcholine, Signalling

1 INTRODUCTION

Proliferation of cultured cells is controlled by growth factors able to elicit the formation of intracellular mitogenic signals through different pathways Likewise, it is commonly accepted that the products of several oncogenes interfere with such pathways both mimicking and substituting for essential components of the signalling cascade. Study of the intracellular second messengers controlling cell proliferation and tumorigenesis is particularly important in the hematopoietic tissue, where growth, differentiation, and leukemogenesis in response to growth factors and oncogenes are strictly interconnected (for review see [1]) However, despite the widespread interest in the processes of normal hematopoiesis and leukemogenesis, most critical mitogenic signalling pathways have commonly been studied in cells of different origin and at various stages of differentiation, thus making it difficult to compare results and to trace a unitary picture of how growth factors and oncoproteins signal In order to circumvene this problem, we decided to study signal transduction in a single hematopoietic cell line that could be transfected with various growth factor receptors or transformed by oncogenes that mimicked growth factor stimulation To this end, we decided to utilize the murine hematopoietic cell line designated 32D.

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This immature, non-tumorigenic myeloid line is strictly dependent on interleukin-3 (IL3) for proliferation and survival [1] IL3 requirement could be abrogated either by oncogene-induced transformation (i e by genes such as *erb*B, *abl*, and *src*[1]) or by introduction of foreign growth factor receptor genes followed by stimulation with the appropriate growth factor Thus, we demonstrated that growth factors as diverse as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and colony stimulating factor-1 (CSF-1) could sustain 32D cell proliferation once the receptor gene had been introduced into cells [2–4] Since such a variety of growth factors and oncoproteins could lead to a similar feature (abrogation of IL3 requirement), we reasoned that common signal transducing elements were activated by IL3, EGF, PDGF, CSF-1, and during oncogene-induced transformation However, it is known that each one of these factors relies on different signal transducing mechanisms to elicit mitogenic responses. EGF and PDGF stimulate the turnover of inositol lipids with formation of diacylglycerol and inositol phosphates (for review see [5]); CSF-1 seems to activate phosphatidylinositol-3 kinase in macrophages and fibroblasts without triggering inositol lipid hydrolysis [6]; IL3, on the other hand, activates protein kinase C (PKC) without increasing inositol lipid turnover [7,8]. The mechanism(s) by which IL3 stimulates PKC has not been clarified in detail so far

In order to establish whether or not common mitogenic signalling pathways were activated by different

growth factors and oncogenes in 32D cells, we studied inositol lipid metabolism, diacylglycerol and phosphatidic acid formation, calcium mobilization and phosphatidylcholine turnover in response to growth factor stimulation in normal cells as well as the steady-state level of these messengers in cells transformed by *erb*B, *abl*, and *src* oncogenes

2 MATERIALS AND METHODS

Radioisotopes were from New England Nuclear IL3 and CSF-1 were from Genzyme, EGF (purified, receptor grade) was from Collaborative Research, PDGF was from Amgen The wild-type murine hematopoietic cell line 32D, transfectants expressing the EGF, α PDGF or CSF-1 (c-*fms*) receptors, and transformants harboring the *erb*B, *abl*, and *src* oncogenes have been described [1–4,9] The level of second messengers (either at steady-state, or following stimulation) was determined in cells prelabelled to equilibrium with [^{3}H]*myo*inositol, [^{3}H]- or [^{14}C]glycerol, [^{32}P]*ortho*phosphate, [*methyl*-^{3}H]- or [*methyl*-^{14}C]choline, following labelling and serum-starvation procedures previously described [10,11] [^{3}H]inositol phosphates were separated by ion-exchange chromatography or by high-pressure liquid chromatography [2–4] [^{3}H]- or [^{14}C]diacylglycerol, and [^{32}P]phosphatidic acid were separated by thin-layer chromatography (TLC) [10,12] Intracellular choline metabolites (i e phosphocholine and choline which make up the largest pool of choline metabolites and are most sensitive to ligand stimulation [13]) were extracted and separated as previously described [10,13] Intracellular free Ca^{2+} levels were measured with the fluorescent Ca^{2+} indicator fura-2 [3] Results of second messenger measurement are expressed as fold variation in each analysed metabolite over that without stimulation, or over that of control (normal) 32D cells when comparing different transformants Incorporation of radioactive labelling in a typical experiment was as follows Diacylglycerol 18417±459 glycerol-containing phospholipids 2494147±104597 (cpm±SE, n=7, of radioactivity associated with diacylglycerol or phospholipids spots on TLC plate) Inositol phosphates 3644±152, inositol-containing phospholipids 34151±1633 (cpm±SE, n=7, of radioactivity recovered in the total inositol lipid fraction, and of an aliquot (100 μl) of the organic phase of modified Folch extraction) Intracellular phosphocholine and choline 2836±309 (cpm±SE, n=5) Phosphatidylcholine 44477±2175 (cpm±SE, n=6, of radioactivity associated with phosphatidylcholine spot on TLC plate) Phosphatidic acid 506±32, total phospholipids 4385±293 (cpm±SE, n=5, of radioactivity associated with phosphatidic acid or phospholipids spots on TLC plates) In order to minimize error due to variability in cell number, labelling procedure and extraction efficiency, the ratios diacylglycerol/phospholipids, inositol phosphates/phosphoinositides, phosphocholine/phosphatidylcholine, and phosphatidic acid/phospholipids were taken into account in making calculations This procedure of result normalization has proven reliable in a variety of experimental conditions involving second messenger measurements [10,11,13–15]

3. RESULTS

Although the mitogenic signalling pathway activated by IL3 in 32D cells is largely unknown, several indications point to protein kinase C as a possible mediator of the IL3 effect on proliferation [7,8]. Since activation of protein kinase C in response to growth factors is believed to be achieved by the generation of diacylglycerol, we measured diacylglycerol and other second messenger formation in response to different agents It is worth noting that the IL3 effect was studied in wild-type 32D cells, whereas the effects of EGF, PDGF, and CSF-1 were studied in transfectants expressing their respective receptor [2–4] Control experiments showed that transfectants expressing foreign growth factor receptors retained their ability to respond to IL3 in the same way as wild-type cells did (not shown) Table I shows that IL3, EGF, and CSF-1 stimulated the formation of diacylglycerol to a similar extent; the rate of diacylglycerol formation was comparable to that reported in fibroblasts stimulated by potent growth factors [13] This effect was dose- and time-dependent, time-course experiments showed that maximal accumulation of diacylglycerol occurred within 10 min from stimulation

The 'classical' route leading to diacylglycerol formation is the receptor-mediated hydrolysis of polyphosphoinositides which yields diacylglycerol and calcium-mobilizing inositol polyphosphates Therefore, we investigated whether IL3 and other factors activated inositol lipid turnover in 32D cells Table I shows that EGF, PDGF, and CSF-1 induced formation of inositol phosphates in 32D cells expressing their respective receptor EGF and PDGF stimulated inositol phosphate generation to a similar extent, thus confirming the hypothesis that both growth factors activate phosphoinositidase C through a similar mechanism [16,17] Analysis by high-pressure liquid chromatography revealed a pattern of inositol polyphosphate formation consistent with previous results obtained with EGF or PDGF stimulation (not shown) Experiments performed in the

Table I

Effect of different growth factors on second messenger formation in 32D cells expressing foreign growth factor receptors

Agonist	Cell line	DAG	InsPs	p-cho	PC	PA	Ca^{2+}
IL3	32D	1 54	1 00	1 89	0 70	1 00	90
EGF	EGFR-32D	1 25	5 40	1 00	1 00	2 93	250 (181)
CSF-1	c-fms-32D	1 63	1 48	1 24	0 90	ND	ND
PDGF	PDGFR-32D	ND	6 41	ND	ND	ND	ND

32D cells were labelled with [^{14}C]- or [^{3}H]glycerol, [^{3}H]*myo*inositol [*methyl*-^{14}C]- or [*methyl*-^{3}H]choline, and ^{32}P as described in section 2 Wild-type 32D cells, or transfectants expressing foreign growth factor receptors were then stimulated with the appropriate growth factor for 10 min Final concentration of growth factors was IL3, 500 U/ml, EGF, 500 ng/ml, CSF-1, 500 U/ml PDGF 100 ng/ml In those experiments where inositol phosphates were measured, lithium chloride (20 mM) was added during incubation with agonists Results are expressed as fold increase over control (unstimulated) cells, and are means of at least 5 experiments, each performed in triplicate samples Results were normalized for the total incorporation of radioactivity in precursor phospholipids as described Intracellular free Ca^{2+} concentration was measured by fura-2 and it is expressed as nM, the corresponding value for unstimulated cells was 89 nM In parentheses is indicated the value obtained when EGF stimulation was performed in the presence of 1 mM extracellular EGTA Abbreviations DAG, diacylglycerol, InsPs, total inositol phosphates, p-cho, intracellular phosphocholine and choline, PC, phosphatidylcholine, PA, phosphatidic acid, ND, not determined.

presence of EGTA in the extracellular medium confirmed that stimulation of inositol lipid turnover was independent of extracellular Ca^{2+} In 32D cells expressing the c-*fms* gene (which codes for the CSF-1 receptor), CSF-1 stimulated inositol lipid turnover to a much lower extent than EGF and PDGF, despite having a potent effect on cell proliferation [4] Analogous experiments performed in NIH/3T3 fibroblasts transfected with c-*fms* gave identical results (not shown) These results sustain the hypothesis that CSF-1 does not activate the 'classical' turnover of inositol lipids [6], even though a small, but significantly reproducible, effect on inositol phosphate formation could be observed IL3, on the other hand, consistently failed to stimulate inositol phosphate generation both in wild-type cells, or in transfectants expressing foreign receptors (Table I) Consistent with the results on inositol lipid turnover, EGF induced a rapid rise in free Ca^{2+} from intracellular stores, whereas IL3 did not affect intracellular free Ca^{2+} concentration neither in the presence nor in the absence of extracellular Ca^{2+} (Table I) Taken together these results indicate that diacylglycerol formation observed in response to IL3 could be ascribed to sources other than inositol lipid hydrolysis

Beside the turnover of inositol lipids, diacylglycerol which activates protein kinase C can be formed through at least two other signalling pathways turnover of phosphatidylcholine (for review see [18]), and synthesis de novo [19,20] Stimulation of the turnover of phosphatidylcholine in response to IL3 has been previously demonstrated in a mast/megakaryocyte cell line [21] In order to assess whether phosphatidylcholine turnover was a general response of hematopoietic cells to IL3, we decided to study the effect of IL3 on the agonist-induced intracellular accumulation of choline and phosphocholine, and on the decrease of phosphatidylcholine. Table I shows that IL3 stimulated the formation of water-soluble choline metabolites together with a significant decrease of the precursor phospholipid, i e phosphatidylcholine Identical results were obtained in 32D cells transfected with the EGF receptor, i e in cells able to couple with inositol lipid turnover, thus indicating that phosphoinositide and phosphatidylcholine turnover in response to different growth factors operated independently in the same cell line Taken together these data led to the conclusion that IL3 stimulated diacylglycerol generation in 32D cells by means of increased phosphatidylcholine turnover, therefore, IL3-induced metabolism of phosphatidylcholine appears to be a general phenomenon in hematopoietic cell lines as well as in other cell types stimulated by appropriate growth factors [13]

CSF-1 is known to stimulate phosphatidylcholine hydrolysis in monocytes without triggering the turnover of inositol lipids [22]. Since we had observed a different pattern (i.e. stimulation of inositol lipid metabolism) in 32D cells transfected with the c-*fms* gene, we wondered whether CSF-1 triggered phosphatidylcholine hydrolysis in 32D cells made to express its receptor Table I shows that CSF-1 also stimulated accumulation of choline metabolites and hydrolysis of phosphatidylcholine, albeit to a lower extent than IL3 As expected, addition of CSF-1 to wild-type 32D cells failed to stimulate both signalling pathways (not shown) These data indicate that CSF-1 was able to couple both to inositol lipid and phosphatidylcholine turnover in 32D cells expressing the c-*fms* gene

EGF, on the other hand, did not seem to stimulate phosphatidylcholine turnover in 32D cells made to express its receptor, thus identifying two separate pathways for diacylglycerol formation in response to agonists in 32D cells. In addition to the hydrolysis of polyphosphoinositides, EGF stimulated the formation of phosphatidic acid (Table I) which can be considered a second messenger able to stimulate cell growth [23,24]. Phosphatidic acid can be formed either from phosphorylation of diacylglycerol, or from synthesis de novo, thus being the precursor of diacylglycerol in phospholipid synthesis [18,19] IL3, despite stimulating diacylglycerol formation, did not induce phosphatidic acid production (Table I), thus suggesting that diacylglycerol formed in response to different growth factors underwent different metabolic processing In fact, a peculiar action of EGF on selective incorporation of phosphate into phosphatidic acid has been observed in other cell systems [25] The pathway of de novo synthesis of diacylglycerol was also investigated by non-equilibrium labelling of cells with radioactive precursors as described [19,20] None of the growth factors tested, however, seemed to significantly stimulate this pathway in 32D cells (not shown)

Cell transformation by oncogenes that code for pro-

Table II

Steady-state levels of phosphocholine, inositol phosphates, and diacylglycerol in 32D cells transformed by *erbB*, *abl*, or *src* oncogenes

32D cell line	p-cho	InsPs	DAG
erbB	4 43	3 85	1 86
abl	2 82	3 47	2 19
src	5 91	1 52	ND

Cell cultures were labelled to equilibrium with [*methyl*-^{14}C]choline, [^{3}H]*myo*inositol, or [^{14}C]glycerol, in serum-containing medium After radiolabelling, cultures were washed and maintained for 4 h in serum-free medium, after which the medium was removed and cells incubated for 1 h in 35-mm dishes containing 1 ml of serum-free medium In experiments designed for measuring InsPs formation, lithium chloride (20 mM) was present during the final incubation Intracellular phosphocholine (p-cho), total inositol phosphates (InsPs), and diacylglycerol (DAG) were extracted and measured as described in section 2 Values obtained for each metabolite were normalized for the amount of radioactivity associated with the precursor phospholipid Data are expressed as fold increase over control (i e normal 32D cells were taken as 1 00), and are means of at least three determinations ND, not determined

teins involved in mitogenic signalling has been associated with multifaceted alterations of intracellular second messengers (for review see [26]) Complex changes involving inositol lipid metabolism and phosphatidylcholine turnover in cells transformed by a variety of oncogenes have been reported [26–28] However, the majority of studies on the subject dealt with mouse fibroblast cell lines [27,28], i e cell lines that mostly utilize inositol lipid turnover for signalling in response to potent growth factors such as PDGF, bombesin, or serum. The case of hematopoietic cells is different, since most naturally-occurring hematopoietic growth factors do not stimulate the hydrolysis of phosphoinositides Thus, having assessed that wild-type 32D cells did not normally use the phosphoinositide pathway, we wondered how transformation by oncogenes that abrogated IL3 dependency could affect the level of those second messengers whose generation had been observed in response to growth factors that also abrogated IL3 dependency once the cells were made to express the appropriate receptor. To this end, we decided to study transformants harboring the *erb*B, *abl*, or *src* oncogenes, i e transforming genes whose products substituted for the activated EGF receptor, or tyrosine kinases, respectively

Normal and transformed cultures were pre-labelled to equilibrium with radioactive glycerol, choline, and inositol; afterwards, they were serum-starved for 4 h followed by 1 h final incubation in serum-free medium. Table II shows that all transformants had elevated levels of water-soluble phosphocholine, inositol phosphates, and diacylglycerol over that of control 32D cells The turnover of inositol lipids was measured both in the presence and in the absence of lithium during the final 1 h incubation, in the absence of lithium, values were significantly lower than in its presence, although they remained higher than those of control cells These data indicate that transformation by *erb*B, *abl*, and *src* oncogenes constitutively increased the turnover of inositol lipids and phosphatidylcholine, thus mimicking the effect of growth factors that exploit such second messenger systems to convey mitogenic signals.

4 DISCUSSION

Control of cell proliferation is exerted by proteins able to stimulate or inhibit cell growth, and a number of intracellular signalling pathways have been described. Knowledge of the signals controlling myeloid cell proliferation may prove important in understanding the mechanism of normal hematopoiesis as well as the escape from growth factor requirement that leads to leukemic transformation. Our results showed that the myeloid 32D cell line was able to utilize different second messenger systems in response to physiological and foreign growth factors It appears that the control on the type of second messenger produced was exerted at the level of growth factor receptor Thus, hematopoietic cells made to express the EGF or the α PDGF receptor responded to such growth factors in the same way as epithelial or mesenchymal cells did when stimulated by EGF or PDGF respectively [5,26] IL3, on the other hand, activated the turnover of phosphatidylcholine similarly to the extent in a mast/megakaryocyte cell line [21], thus substantiating the hypothesis that phosphatidylcholine turnover in response to interleukins was a common feature of hematopoietic cells [29]

The effect of CSF-1 on signalling in c-*fms*-expressing cells was rather intriguing: in fact, earlier studies reported that CSF-1 was not able to stimulate the 'classical' turnover of inositol lipids either in macrophages that endogenously express its receptor or in BALB/c fibroblasts made to express the receptor [6] However, overexpression of CSF-1 receptor in 32D cells and in NIH/3T3 fibroblasts [4] led us to observe a small, but significantly reproducible, accumulation of inositol phosphates in response to the factor (Table I). Several explanations may account for this phenomenon The CSF-1 receptor gene product shares structural and sequence similarities with the PDGF receptor however, while tyrosine kinase domains are very similar, their kinase insert domains are highly unrelated both in predicted sequence and length In a recent study we demonstrated that insertion of the c-*fms* kinase insert domain could reconstitute biochemical and biological responses to PDGF, in 32D cells expressing a deletion mutant of the α PDGF receptor lacking the original kinase insert [9] Using a similar approach we also demonstrated that tyrosine mutations within the α PDGF receptor kinase insert domain could abrogate receptor-associated phosphatidylinositol-3 kinase activity without affecting phosphoinositide turnover and mitogenic and chemotactic signal transduction [30] These results support the consideration that stimulation of inositol lipid metabolism appears to be a mandatory event in PDGF signalling [30,31] Therefore, it is proposed that the overexpressed c-*fms* product might interact with, and stimulate with low efficiency, the PDGF signalling pathway, thus leading to inositol phosphate formation Future studies will determine whether the phosphoinositidase C expressed in 32D cells is a good substrate for CSF-1 receptor tyrosine kinase In addition to the hydrolysis of phosphoinositides, CSF-1 also stimulated the turnover of phosphatidylcholine; these results are consistent with a previous observation made in human monocytes [22], thus indicating that phosphatidylcholine turnover in response to CSF-1 was a feature shared by different hematopoietic cell lines From these results, however, we cannot conclude whether CSF-1 signalled through both pathways, or whether stimulation of inositol lipid turnover was just an epiphenomenon due to overexpression of the receptor.

Finally, transformation by oncogenes the products of which mimicked growth factor signalling, at different

levels, was associated with elevated turnover of inositol lipids and phosphatidylcholine; these results suggest that the same pathways activated by growth factors were constitutively operating during transformation. It is worth noting that these results were obtained in a hematopoietic cell line which is devoid of growth factor receptors coupling to inositol lipid turnover; therefore, alteration of phosphoinositide metabolism could be ascribed to a direct effect of oncogene-induced transformation, ruling out at the same time 'cross-talk' between endogenous receptors coupling with inositol lipid turnover and oncoproteins Furthermore, 32D cells provided an example of transformed cells utilizing both phosphoinositide and phosphatidylcholine turnover, these results are at variance with those obtained in 3T3 fibroblasts, where the two pathways appeared mutually exclusive [10,11,20,26] From what is reported above, it follows that the major point of convergence of the different signalling pathways activated in response to growth factors, and during transformation, was diacylglycerol formation, and, therefore, protein kinase C activation.

In conclusion, we assessed that different signalling pathways were available in myeloid 32D cells to control proliferation, the type of mitogenic signalling pathway was dependent on receptor expression Transformation by *erb*B, *abl*, and *src* oncogenes was associated with complex alterations of signal transduction involving both inositol lipid and phosphatidylcholine turnover Our data suggest that diacylglycerol formation, and protein kinase C activation, could play a prominent role in the abrogation of growth factor requirement in myeloid cells.

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