p44/42 mitogen-activated protein kinase is involved in the expression of ornithine decarboxylase in leukaemia L1210 cells

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The involvement of p44/42 mitogen-activated protein kinase (MAPK) in the induction of ornithine decarboxylase (ODC) was investigated by using PD98059, a specific MAPK-kinase (MEK1/2) inhibitor, and other signal-transduction inhibitors. In D,L-α-difluoromethylornithine (DFMO)-resistant L1210 cells stimulated to grow from quiescence, treatment with PD98059 inhibited p44/42 MAPK phosphorylation and the induction of ODC activity and protein. A marked reduction of the accumulation of mature ODC mRNA and its intron-containing precursor was observed, whereas ODC turnover was hardly affected. PD98059 also reduced the content of antizyme, but not that of antizyme mRNA. U0126, a novel and more potent inhibitor of MEK1/2, provoked a dose-dependent inhibition of ODC induction at lower concentrations with respect to PD98059. Other effective inhibitors of ODC induction proved to be genistein, manumycin A, herbimycin A, LY294002, wortmannin and KT5823, suggesting the involvement of other key proteins of signal-transduction pathways, i.e. Ras, Src, phosphatidylinositol 3-kinase and cGMP-dependent protein kinase, which may have a positive impact on MAPK. Cells kept in a DFMO-free medium, and thus containing high levels of putrescine and spermidine, showed enhanced MAPK phosphorylation and lower sensitivity to PD98059, compared with cells maintained in the presence of DFMO. In conclusion, these results indicate that the activation of p44/42 MAPK may favour the expression of ODC, and that polyamines, in turn, may affect the phosphorylation state of MAPK.

Key words: antizyme, PD98059, phosphatidylinositol 3-kinase, polyamines, signal transduction.

INTRODUCTION

Stimulation of cultured cells with serum or purified growth factors results in the triggering of multiple signal-transduction pathways, eventually leading to cell proliferation. These pathways are initiated by receptor tyrosine kinases or receptor-recruited tyrosine kinases, which create binding sites for proteins containing Src homology 2 (SH2) domains [1]. Src homology 2-domain-containing proteins include: adaptor proteins, such as SHC or GRB2, which lead to the activation of the Ras/mitogen-activated protein kinase (MAPK) cascade; phospholipase C-γ, which leads to the activation of diacylglycerol-dependent protein kinase C (PKC) isoforms; p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K); and Src, a tyrosine kinase that may contribute to the activation of both PI3K and MAPK cascades. Co-ordination and integration of these signal-activated routes may require an intricate cross talk among their constituents.

The Ras/MAPK cascade is the best-defined pathway involved in cell proliferation and can transfer the information to the nucleus affecting the activity and/or the expression of critical transcription factors [1]. In this pathway, a central role is played by p44 and p42 MAPKs [also known as extracellular-signal-regulated kinase (ERK) 1 and ERK2, respectively], which are activated through phosphorylation at the sequence T*EY* (where * denotes a phosphorylated residue) by a single type of dual-specificity MAPK kinase (MEK1/2) that in turn can be activated following the sequential activation of Ras and Raf. The compound PD98059 [2], recently available, acts by binding the inactive form of MEK1/2 and blocking its activation, and represents a very specific and now widely used tool to dissect the MAPK pathway and identify its downstream targets.

Growing evidence suggests that polyamines are involved intimately in the control of cell proliferation and even in the development of cancer. Ornithine decarboxylase (ODC), the first and rate-limiting enzyme in polyamine biosynthesis, is induced following growth stimuli [3–5]. The *ODC* gene is now recognized as a proto-oncogene required for cell-cycle progression and transformation [4,6]. ODC is one of the most highly regulated enzymes known: its expression can be controlled at multiple levels and a specific inhibitor to ODC, named antizyme, has been described and cloned [3–5].

Although it has long been known that ODC can be induced in cells stimulated from quiescence by serum or growth factors, the signal-transduction pathways involved are scarcely defined. In particular, to our knowledge, no definitive evidence is available about the involvement of MAPK in ODC induction. In fact, protein tyrosine kinase inhibitors, such as genistein and herbimycin A, have proved effective in reducing ODC activity induced by a few stimuli [7–13]. In hepatocyte growth factor-treated HepG2 cells, however, genistein can exert stimulating effects on ODC activity and mRNA [14]. Previous work has shown that cell transformation induced by the oncogenes v-*src* or c-Ha-*ras* is associated with constitutively elevated ODC activity and mRNA [15–17]. According to a recent report [18], however, ODC activity in v-*raf*-transformed cells is about 10-fold lower

Abbreviations used: ODC, ornithine decarboxylase; DFMO, D,L-α-difluoromethylornithine; L1210-DR, DFMO-resistant L1210; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; RT, reverse transcriptase.

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than in cells tranformed by *ras*. Moreover, constitutive modulation of Raf-1 has been found to be positively associated with differential expression of the antizyme gene [19]. Although these findings suggest the importance of protein tyrosine phosphorylation for ODC induction and the involvement of Ras and Src, it should be noted that Src, Ras and also Raf may have downstream targets other than MAPK [1,20]. For example, both Src and Ras can have a positive impact on PI3K, which in turn may produce effects even independently of the MAPK pathway [21]. In this regard, quite recently we have shown that PI3K activity is required for ODC induction in D,L-α-difluoromethylornithine (DFMO)-resistant L1210 (L1210-DR) cells stimulated to growth [22]. Therefore, in the present study, we have investigated the requirement of the MAPK pathway for ODC induction and expression by using the highly specific MEK inhibitor PD98059 and other signal-transduction inhibitors. The effects on antizyme expression were also reported. Most of the experiments were carried out with L1210-DR cells, which have been selected for resistance to the ODC inhibitor DFMO [23]. Because of gene amplification, these cells can express ODC at high levels and represent a useful model to compare the effects of high and low levels of polyamines, when grown in the absence or presence of DFMO, respectively.

EXPERIMENTAL

Materials

L1210-DR cells [23] and anti-mouse ODC antibody were a generous gift of Dr. L. Persson (University of Lund, Lund, Sweden). Anti-recombinant rat antizyme antibody [24] was provided generously by Dr. S. Matsufuji (Jikei University School of Medicine, Tokyo, Japan). Anti-p44/42 MAPK and anti-phospho-specific p44/42 MAPK antibodies were purchased from New England Biolabs. DFMO was provided kindly by the Merrell Dow Research Institute (Strasbourg, France). PD98059 and other inhibitors of signal-transduction pathways were purchased from Alexis, except manumycin A and KT5823 (from Calbiochem), U0126 (from Promega) and wortmannin (from Sigma). Oligonucleotide primers for reverse transcriptase (RT)-PCR were either synthesized with an ABI 391 DNA synthesizer (Applied Biosystems) and purified by HPLC or (in the case of the primers for antizyme) purchased from Gibco-BRL.

Cell culture and treatments

Mouse L1210-DR cells were grown routinely as described previously [23,25] and kept in the presence of 20 mM DFMO or in a DFMO-free medium for at least 10 days. For experiments, quiescent cells (from 4–5-day-old cultures; cell density $\geqslant 2\times10^6$/ml) were seeded at $2–3\times10^5$/ml in fresh medium containing serum. Unless stated otherwise, PD98059 and other inhibitors were added to quiescent cells 30 min before seeding (60 min in the case of manumycin A) and again at cell dilution to keep the indicated concentrations. However, PD98059 was equally effective in reducing ODC induction when added only at seeding or 10 min after seeding. The inhibitors were added in DMSO (final concentration 0.1 %). Control cells received equal amounts of the vehicle. Wild-type L1210 cells were utilized under the same experimental conditions. Cell viability was checked by Trypan Blue exclusion. At the time indicated after seeding, cells were harvested and washed with PBS. Unless stated otherwise, the data shown in the Figures come from one experiment representative of two or more experiments.

Determination of ODC activity and polyamine content

Cell extracts were prepared and assayed for ODC activity as described previously [25]. Specific ODC activity is expressed as units/mg of protein, where 1 unit corresponds to 1 nmol of CO_2/h of incubation. Polyamines (from at least 2×10^6 cells) were analysed in acid extracts by HPLC, after derivatization with dansyl chloride [26].

Western-blot analyses

Western-blot analysis of ODC was carried out as described previously [22]. Western blotting of antizyme was performed essentially as for ODC, but 80 μg of protein were subjected to SDS/PAGE (15 % gel). In the case of p44/42 MAPK, about 10^7 cells were resuspended in 0.1 ml of lysis buffer [20 mM Tris/HCl (pH 8)/100 mM NaCl/5 mM EDTA/1 mM Na_3VO_4/1 mM benzamidine/1 % Nonidet P40/1 mM PMSF/10 mM *p*-nitrophenylphosphate/1 mM dithiothreitol/10 mM β-glycerophosphate/1 μg/ml aprotinin/1 μg/ml leupeptin/1 μg/ml pepstatin], sonicated and centrifuged. The supernatant was boiled in loading buffer and an aliquot corresponding to 60 μg of protein was analysed using SDS/PAGE (12 % gel). Separated proteins were transferred on to a nitrocellulose membrane for 1 h. The membrane was saturated with 4 % powdered milk/0.05 % Tween 20 in 10 mM Tris (pH 8)/150 mM NaCl for 1 h, and then incubated with either control anti-p44/42 MAPK antibody or anti-phospho-specific p44/42 MAPK antibody at 4 °C overnight. Bands were revealed by the Amersham ECL detection system.

Detection of mRNAs

mRNAs were detected in L1210-DR cells by RT-PCR analysis. Total RNA was isolated from about 10^7 cells by the TriPure Isolation Reagent (Boehringer Mannheim) according to the manufacturer's instructions. Any contaminating DNA was removed by DNase treatment and then RNA aliquots (0.25 μg) were reverse-transcribed, amplified for 20–30 cycles and analysed by agar-gel electrophoresis as described previously [22,27]. Specific primers were designed on the basis of published sequences as follows. For mature ODC mRNA, the 5′ primer was targeted to a sequence in exon 9 of mouse ODC mRNA and the 3′ primer recognized a sequence at the junction of exons 10 and 11, yielding a PCR product of 224 bp [27]. For ODC pre-mRNA, the 5′ and 3′ primers were targeted to sequences of introns 8 and 9 respectively, yielding a PCR product of 247 bp [27]. For mature antizyme mRNA, the 5′ primer (5′-AAGGACAGTTTTGCA-GCTCT-3′) recognized a sequence at the junction of exons 3 and 4 and the 3′ primer (5′-CATGAAGCAAGCGTCGGGTCTC-TT-3′) was targeted to a sequence in exon 5, yielding a PCR product of 186 bp. For β-actin mRNA, used as a control, primers were as described previously [28], yielding a 214-bp product. The number of PCR cycles and the amount of total RNA added were chosen in the range of proportionality of PCR amplification.

RESULTS

Effect of PD98059 and other signal-transduction inhibitors on p44/42 MAPK activation and ODC induction

L1210-DR cells were stimulated to proliferation by dilution of high-density quiescent cells in fresh medium containing serum

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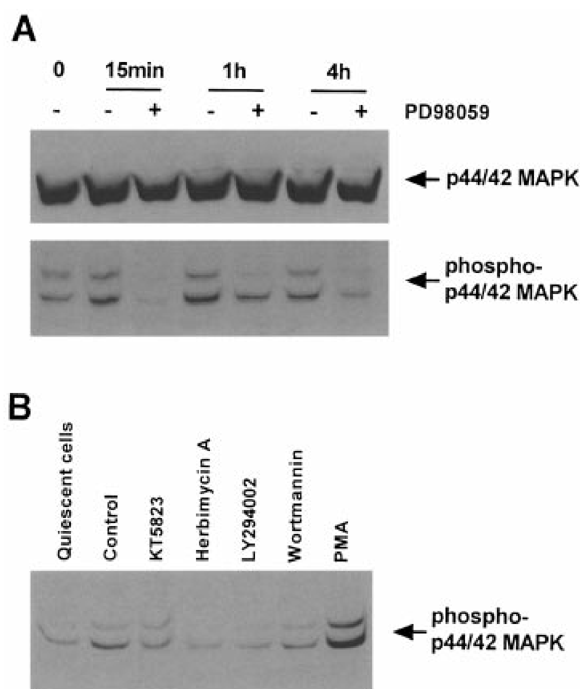

Figure 1 p44/42 MAPK is activated by phosphorylation in L1210-DR cells stimulated to growth: effect of PD98059 and other signal-transduction inhibitors

(**A**) Quiescent cells were stimulated to growth by dilution in fresh medium containing serum and harvested at the times indicated. Incubation with PD98059 (50 μM) started 30 min before cell dilution, as detailed in the Experimental section. Cell extracts were analysed by Western blotting by using control p44/42 MAPK or phospho-specific p44/42 MAPK antibodies. (**B**) Quiescent cells were treated with the indicated compounds (concentrations were as reported in Table 1) and harvested 15 min after stimulation to growth. The effect on p44/42 MAPK phosphorylation is shown. Total MAPK was not changed significantly by the various agents (results not shown).

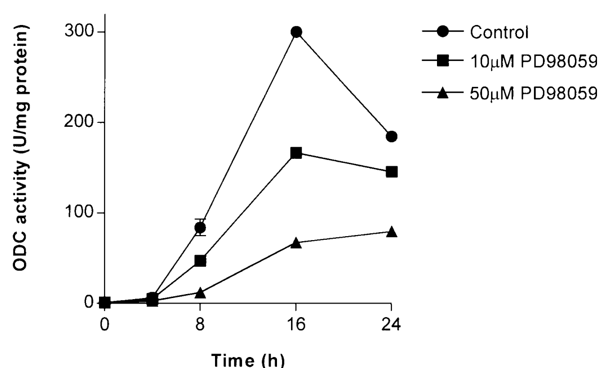

Figure 2 PD98059 inhibits the induction of ODC activity in L1210-DR cells stimulated to growth

Cells were treated as described in the legend of Figure 1, harvested at the times indicated after cell dilution and assayed for ODC activity. Results are means ± S.D. U, units.

[22,25,27]. p44/42 MAPK was expressed in these cells and its content did not vary significantly following cell stimulation (Figure 1A); instead, this procedure resulted in a rapid increase of active phosphorylated p44/42 MAPK, more evident in the case of the p42 isoform. Phosphorylation was maximal at 1 h, declined after 4 h (Figure 1A), and returned to low levels after 8 h (results not shown). The MEK1/2 inhibitor PD98059 inhibited this increase markedly, even if the inhibition was not complete over all the time examined. Figure 2 shows that the induction of ODC activity followed p44/42 MAPK activation with a peak at 16 h, and PD98059 treatment reduced the increase of ODC activity dose-dependently, particularly at 8 and 16 h. PD98059 provoked inhibition of the induction of ODC activity, even in wild-type L1210 cells (by about 70 % at 8 and 16 h in the presence of 50 μM PD98059; results not shown), indicating that this effect is not limited to a particular ODC-overproducing cell line.

Table 1 Effects of various inhibitors of signal-transduction pathways on the induction of ODC activity in L1210-DR cells

Quiescent cells were treated with the indicated compound and stimulated to growth. After 8 h, cells were harvested and assayed for ODC activity. Results are means ± S.D. PKG, cGMP-dependent protein kinase.

Agent	Specificity	Concentration	ODC activity (% of control)
PD98059	MEK1/2 inhibitor	10 μM	46.7 ± 3.6
		50 μM	14.2 ± 2.7
U0126	MEK1/2 inhibitor	1 μM	10.5 ± 3.5
		10 μM	3.5 ± 0.6
Manumycin A	Ras farnesylation inhibitor	10 μM	24.7 ± 9.1
Genistein	Broad-range tyrosine kinase inhibitor	100 μM	2.6 ± 1.0
Herbimycin A	Src-family tyrosine kinase inhibitor	1 μM	38.3 ± 5.2
LY294002	PI3K inhibitor	20 μM	1.2 ± 1.0
KT5823	PKG inhibitor	1 μM	54.3 ± 9.5
Wortmannin	PI3K inhibitor	100 nM	23.7 ± 5.8
Chelerythrine	PKC inhibitor	1 μM	89.9 ± 0.6
PMA	PKC activator	100 ng/ml	17.7 ± 0.4

In addition to PD98059, several inhibitors of signal-tranduction pathways endowed with various specificities were tested for their ability to affect the induction of ODC activity in L1210-DR cells. These results are shown in Table 1. U0126, a novel and more potent inhibitor of MEK1/2 [29] than PD98059, provoked a dose-dependent inhibition of ODC induction at significantly lower concentrations than did PD98059. In fact, an inhibition by about 90 % was obtained at an at-least 50-fold lower concentration. Genistein, a broad-range tyrosine kinase inhibitor, and manumycin A, an inhibitor of Ras farnesylation [30], were also effective, supporting the involvement of tyrosine phosphorylation and Ras in ODC induction. Other inhibitors of the induction of ODC activity proved to be herbimicyn A, a tyrosine kinase inhibitor of the Src family, the PI3K inhibitors LY294002 and wortmannin, as reported previously [22], and KT5823, a specific inhibitor of cGMP-dependent protein kinase (PKG) [31]. These inhibitors also reduced p44/42 MAPK phosphorylation (Figure 1B), indicating that Src, PI3K and PKG may utilize, at least in part, MAPK as a downstream effector to induce ODC. In contrast, chelerythrine, considered to be a specific PKC inhibitor [32], was hardly effective at 1 μM (higher concentrations were toxic for the cells), and PMA, a well-known PKC activator, actually reduced ODC activity (Table 1). The PMA effect was remarkable after only 4 h and observed even in the absence of serum (results not shown). It should be noted that PMA treatment increased MAPK phosphorylation further with respect to control stimulated cells (Figure 1B). Thus these data suggest that, in this experimental model, PMA and PKC may have other targets in addition to MAPK, which lead to a reduction of ODC induction.

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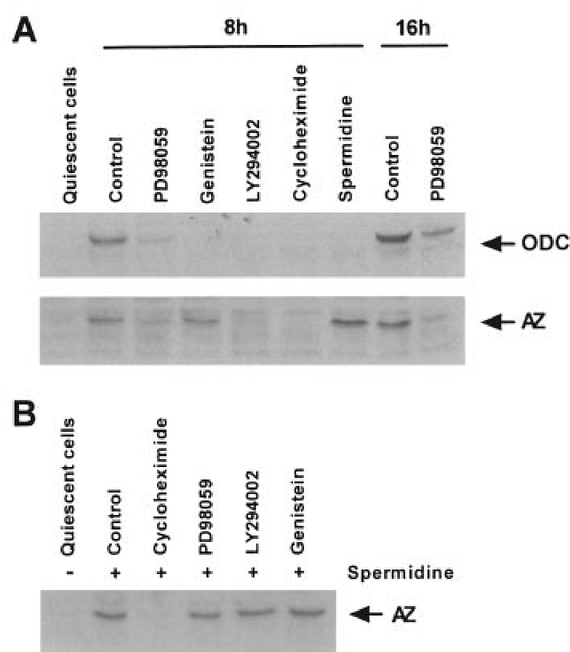

Figure 3 Effect of PD98059 and other inhibitors on the induction of ODC and antizyme (AZ) protein analysed by Western blotting

(**A**) Cells were treated with the indicated compounds and harvested 8 or 16 h after stimulation to growth. PD98059 and spermidine concentrations were 50 μM and 1.5 mM, respectively. Concentrations of genistein and LY294002 were as in Table 1. Cycloheximide (0.2 mM) was added only 3 h before harvesting. (**B**) Cells were treated as in (**A**) except that 1.5 mM spermidine was added at cell dilution to all samples. After 8 h cells were harvested.

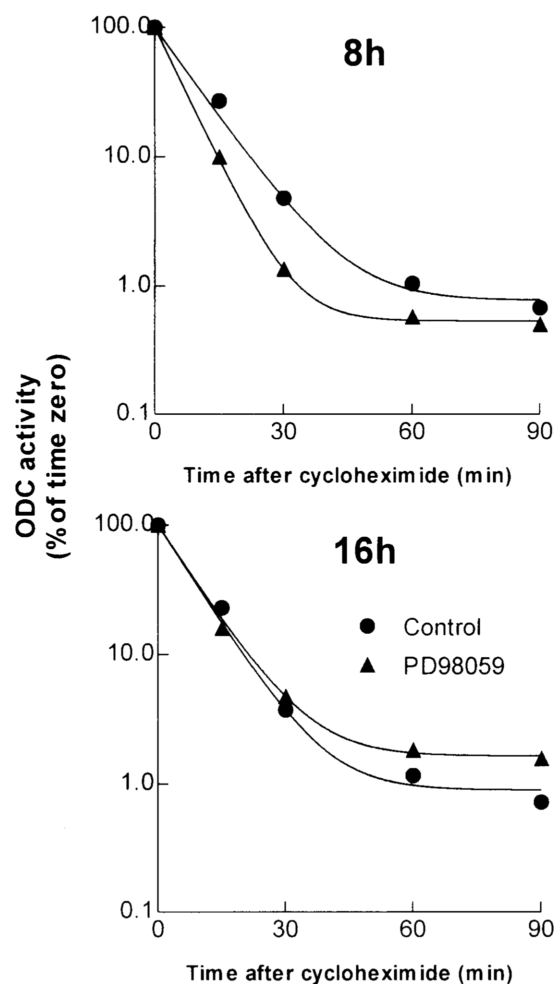

Figure 4 Effect of PD98059 on the rate of ODC decay

Quiescent cells were treated with 50 μM PD98059 (▲) or not (control, ●) and stimulated to growth for 8 or 16 h. Then, 0.2 mM cycloheximide was added (time 0) and samples were removed at the times indicated. $t_{\frac{1}{2}}$ values were 6.5 and 4.3 min for control and PD98059-treated cells (8 h), and 5.9 and 6.0 min for control and PD98059-treated cells (16 h), respectively.

Since ODC can be controlled by antizyme, a protein inhibitor specific to ODC [5], we have examined the effects of PD98059 and other kinase inhibitors on the contents of ODC and antizyme protein. Western-blot analysis of ODC (Figure 3A) shows that the induction of ODC protein was reduced strongly by PD98059, at both 8 and 16 h after cell stimulation. Moreover, the ODC band virtually disappeared following genistein and LY294002. Thus the levels of ODC protein appear to parallel those of ODC activity. Antizyme was identified by immunoblotting with a specific antibody [24] as a band that disappeared after a 3-h treatment with cycloheximide, as expected from its rapid turnover, and increased after spermidine (Figure 3A). Antizyme level, low in quiescent cells, augmented after cell stimulation (at 8 h and even more at 16 h) and treatment with PD98059 or LY294002 reduced this induction significantly. This effect may be, at least in part, secondary to inhibition of ODC induction and consequent polyamine accumulation, which is known to induce antizyme at the translational level [5]. In accordance with this hypothesis, the reduction of antizyme level following these kinase inhibitors was hardly seen or less evident when the experiment was performed in the presence of exogenously added spermidine (Figure 3B). On the other hand, genistein did not reduce antizyme levels remarkably compared with control cells (Figure 3A), or in some experiments genistein reduced antizyme levels only slightly, even if ODC activity and protein disappeared. The reason for these discrepancies remains to be determined.

Effect of PD98059 on ODC turnover and expression

As an important point of control of ODC is at the level of protein degradation, ODC turnover was evaluated following block of protein synthesis with cycloheximide. The rate of ODC decay increased slightly in PD98059-treated cells after 8 h (the half-life time decreased by about 35% with respect to control cells), and it did not change significantly after 16 h (Figure 4). Since the rate of ODC turnover and degradation is thought to vary as a function of the ODC/antizyme ratio [5], the lack of, or only minor changes in, ODC turnover following PD98059 may be consistent with the reduction in both ODC and antizyme levels, noted above (Figure 3A).

Since the effects on ODC turnover cannot explain the strong reduction of ODC protein by PD98059, an effect on ODC synthesis may be inferred. ODC can be controlled at different levels of gene expression, including transcription [3,4], thus the effects of PD98059 (and genistein for comparison) on ODC mRNA expression were examined by detection of mature ODC mRNA and its intron-containing pre-mRNA precursor (Figure 5). Quiescent cells show low levels of both mature and pre-mRNA for ODC, which were enhanced following cell stimulation, and the treatment with PD98059 or genistein reduced or prevented this accumulation. These data suggest that PD98059 and genistein actions may be mediated at least partially at the

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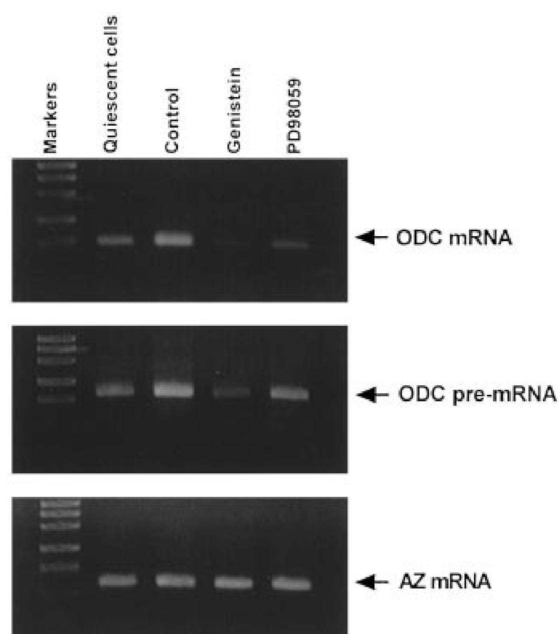

Figure 5 Effect of PD98059 and genistein on the expression of mature ODC mRNA, its precursor (ODC pre-mRNA) and antizyme (AZ) mRNA analysed by RT-PCR

Quiescent cells were treated with nothing (control), 100 μM genistein or 50 μM PD98059, stimulated to growth and harvested after 8 h. DNA molecular-size markers were (from top): 517, 453, 394, 298, 234, 220 and 154 bp.

Table 2 Effect of DFMO and PD98059 on polyamine content of L1210-DR cells

Quiescent cells, cultivated routinely in either the presence or absence of DFMO, were treated or not with 50 μM PD98059, and harvested 16 h after cell stimulation. Results represent means $\pm$ S.D.

		Polyamine content (nmol/mg of protein)		
Cell condition	Inhibitor	Putrescine	Spermidine	Spermine
L1210-DR	–	34.1 ± 1.2	23.1 ± 0.6	7.9 ± 0.3
L1210-DR	PD98059	16.7 ± 0.6	22.8 ± 0.1	7.1 ± 0.4
L1210-DR + DFMO	–	1.9 ± 0.4	14.7 ± 0.8	10.8 ± 2.5
L1210-DR + DFMO	PD98059	0.75 ± 0.2	13.2 ± 0.9	9.5 ± 0.8

transcriptional level. It should be noted that, under the same conditions, antizyme mRNA levels were not affected significantly (Figure 5), in accordance with the notion that antizyme expression is regulated mainly at the translational level [5]. The content of β-actin mRNA, used as a control, was also not changed by these drugs (results not shown).

Effect of DFMO on MAPK phosphorylation

In order to study the interrelationship between polyamines and MAPK, we have utilized L1210-DR cells grown either in the presence or absence of DFMO, as a model to compare the effects of high and low levels of polyamines. Table 2 shows that cells grown in a DFMO-free medium contain very high amounts of putrescine and spermidine in agreement with previous data [23].

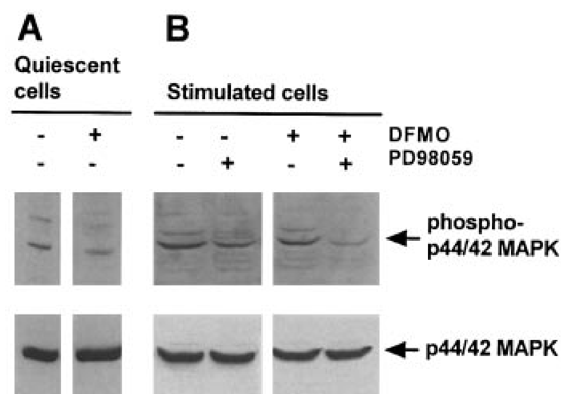

Figure 6 Effect of DFMO on the phosphorylation state of p44/42 MAPK

L1210-DR cells grown routinely in either the presence or absence of DFMO were analysed by Western blotting as described in the legend of Figure 1. (**A**) Quiescent cells; (**B**) quiescent cells were treated or not with 50 μM PD98059 and harvested 1 h after stimulation to growth. The results shown in (**A**) and (**B**) are from two separate experiments.

On the other hand, cells maintained in the presence of DFMO show a detectable, but much lower, level of putrescine and a lower content of spermidine which, however, allow resistance to DFMO and cell growth [23]. After stimulation to growth for 16 h, PD98059-treated cells exhibited a lower content of putrescine with respect to control cells.

Next we compared the phosphorylation state of MAPK in L1210-DR cells grown in the presence or absence of DFMO. In quiescent cells, the degree of MAPK phosphorylation appeared lower in cells with DFMO (Figure 6A). After cell stimulation to growth, MAPK phosphorylation was still lower in cells kept in the presence of DFMO; however, the difference was more evident in the case of PD98059 treatment (Figure 6B). It should be noted that the total amount of MAPK was not affected significantly by the presence of DFMO. Interestingly, L1210-DR cells grown in DFMO-free medium were less sensitive to inhibition of cell proliferation by PD98059 with respect to cells grown routinely in the presence of DFMO. In fact, the number of cells at day 2 after cell stimulation was reduced to 55 % of control by 50 μM PD98059 when the cells were grown in the presence of DFMO and only to 91 % when cells were kept in the absence of the ODC inhibitor (results not shown).

DISCUSSION

Inhibitor studies with genistein, a broad-range tyrosine kinase inhibitor, have generally shown the relevance of tyrosine phosphorylation for ODC induction. Genistein has been reported to prevent ODC induction by a phorbol ester [8] and some agonists of seven-membrane-spanning receptors [9–11] or T-cell-antigen receptors [7]. Interestingly, Tseng and Verma [8] reported that genistein could inhibit PMA-induced ODC activity and MAPK activation in monkey kidney epithelial CV-1 cells. These authors, however, also found that genistein reduced PMA-stimulated phosphorylation of p70 S6 kinase, a kinase that lies in a MAPK-independent pathway, at concentrations lower than that effective in inhibiting MAPK activation [8]. The possibility that PMA may regulate ODC induction, even through MAPK-independent pathways, is also shown by the present study. In fact, although PMA further increased p44/42 MAPK phosphorylation of stimulated L1210-DR cells, the induction of ODC was decreased markedly. In accordance with our results,

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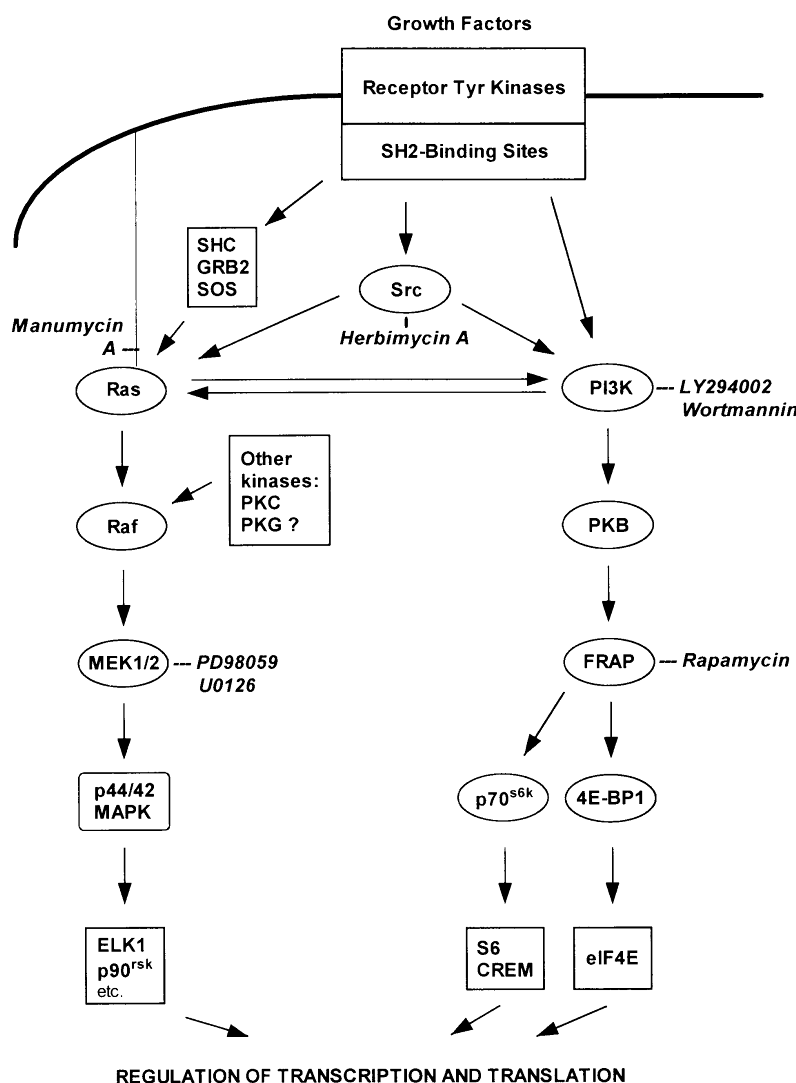

Scheme 1 Position of some key proteins in signalling pathways and their inhibitors

The Scheme, drawn according to current knowledge, is simplified. In particular, Src may act on more than a step of the Ras pathway (e.g. by phosphorylating SHC and Raf). Besides, Ras, Src, Raf, PI3K and protein kinase B (PKB) may have other targets in addition to those indicated. Abbreviations not in the text: FRAP, FK506-binding-protein-rapamycin-associating protein; eIF, eukaryotic initiation factor; 4E-BP, eIF4E-binding protein; CREM, cAMP-response-element modulator; SH2, Src homology 2; p70^{s6k}, p70 S6 kinase; p90^{rsk}, p90 ribosomal S6 kinase; S6, a ribsomal protein; Tyr, tyrosine.

Ruhl et al. [33] have shown that PMA can cause a marked reduction in ODC enzyme activity in human keratinocytes through a post-transcriptional mechanism. Moreover, according to a recent paper, genistein can exert stimulating effects on ODC activity and mRNA in hepatocyte growth factor-treated HepG2 cells [14]. In the present study with leukaemia L1210-DR cells, genistein strongly inhibited the induction of ODC protein and the expression of its messenger. In addition, a role for antizyme may not be excluded. However, the effect of genistein may result from inhibition of several signalling routes, since this drug can inhibit receptor and non-receptor tyrosine kinases.

The use of more specific inhibitors shows that multiple pathways may be required for the induction of ODC in this experimental model. These may include MAPK-, Ras-, Src- and PI3K-dependent pathways. To our knowledge, this study is the first showing the involvement of p44/42 MAPK in ODC induction and expression on the basis of the effects of specific MEK1/2 inhibitors. Although PD98059 and U0126 appear to exhibit similar mechanisms for inhibiting MEK, U0126 shows a mode of inhibition in part distinct from PD98059 and a much higher affinity for MEK [29]. Accordingly, U0126 was able to inhibit ODC induction at significantly lower concentrations than PD98059. The experiments with PD98059 suggest a possible action at the transcriptional level. In this regard, a number of *cis*- and *trans*-activating factors of the *ODC* gene have been identified ([34,35] and references therein), but their relevance in mediating a possible p44/42 MAPK-dependent transcriptional stimulation remains to be assessed. In addition, Src and PI3K inhibitors can have a negative impact on p44/42 MAPK in L1210-DR cells, thus explaining at least part of the ODC-inhibiting effect of these compounds. However Ras, Src and PI3K could also affect p44/42 MAPK-independent targets [1,18,20,21], which may support ODC induction, particularly at the translational level. In this regard, a rapamycin-sensitive pathway may play an im-

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portant role [22,36,37]. Moreover, our results with KT5823 suggest that PKG is also required for maximal ODC induction and MAPK activation. Accordingly, in colecystokinin-stimulated Chinese hamster ovary cells, proliferation and MAPK activation have been found to depend on the cGMP-dependent pathway [31]. A simplified scheme depicting some pathways discussed in the present study is shown in Scheme 1.

Antizyme expression is known to be controlled mainly by polyamines, through a translational frameshift of its messenger [5]; however, Yang et al. [38] have found recently that interleukin-1 can elevate antizyme gene transcription in human melanoma cells. Furthermore, Patel et al. [19], using differential display of mRNA, identified antizyme as a gene overexpressed in human squamous carcinoma cells transfected with *raf-1*. In the present study, PD98059 treatment reduced the content of antizyme protein in the absence, but not in the presence, of exogenously added polyamines, and did not affect the level of antizyme mRNA, indicating that MAPK is not involved in the transcriptional control of the antizyme gene.

The importance of ODC induction and consequent polyamine accumulation for MAPK-supported cell proliferation seems to be indicated by the fact that L1210-DR cells, grown in DFMO-free medium, were less sensitive to inhibition of cell proliferation by PD98059 with respect to cells grown routinely in the presence of DFMO. This may be due to the very high levels of putrescine and spermidine (Table 2 and [23]): thus even if ODC induction is inhibited by PD98059, the amounts of putrescine and spermidine remain elevated and may circumvent the inhibition of MAPK, which however is not complete. On the other hand, some authors have reported that ODC and polyamines may have a positive impact on MAPK in some experimental models [39–42]. However, other reports are not in agreement with these findings [18,43]. In our experimental conditions, the high levels of putrescine and spermidine present in the absence of DFMO seem to have a positive effect on MAPK phosphorylation and make MAPK less sensitive to PD98059 inhibition, as judged by the comparison of cells kept with or without DFMO (Figure 6). Bachrach and Tabib [41] have also reported that addition of polyamines to NIH 3T3 cells appears to reverse PD98059's inhibiting effects of MAPK phosphorylation as well as of c-*myc* and c-*fos* expression.

In conclusion, the present results suggest that polyamines may be located both downstream and upstream of p44/42 MAPK and hint at the existence, under some circumstances, of a positive loop involving ODC and MAPK (MAPK → ODC → polyamines → MAPK), which could be relevant for the 'proto-oncogene' function of ODC.

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