Transcriptional and translational control of ornithine decarboxylase during Ras transformation

Lisa M. SHANTZ¹

Department of Cellular and Molecular Physiology, The Milton S. Hershey Medical Center, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033, U.S.A.

ODC (ornithine decarboxylase) activity is induced following *ras* activation. However, the Ras effector pathways responsible are unknown. These experiments used NIH-3T3 cells expressing partial-loss-of-function Ras mutants to activate selectively pathways downstream of Ras and examined the contribution of each pathway to ODC induction. Overexpression of Ras12V, a constitutively active mutant, resulted in ODC activities up to 20-fold higher than controls. Stable transfections of Ras partial-loss-of-function mutants and constitutively active forms of MEK (MAPK kinase) and Akt indicated that activation of more than one Ras effector pathway is necessary for the complete induction of ODC activity. The increase in ODC activity in Ras12V-transformed cells is not owing to a substantial change in ODC protein half-life, which increased by < 2-fold. Northern-blot analysis and reporter assays suggested that the mechanism of ODC induction involves both a modest increase in the transcription of ODC

mRNA and a much more considerable increase in the translation of mRNA into protein. ODC transcription was controlled through a pathway dependent on Raf/MEK/ERK (where ERK stands for extracellular-signal-regulated kinase) activation, whereas activation of the phosphoinositide 3-kinase and the Raf/MEK/ERK pathways were necessary for translational regulation of ODC. The increase in ODC synthesis was accompanied by changes in phosphorylation of eukaryotic initiation factor 4E and its binding protein 4E-BP1. Results show that the phosphoinositide 3-kinase pathway regulates phosphorylation of both proteins, whereas the Raf/MEK/ERK pathway affects only the eukaryotic initiation factor 4E phosphorylation.

Key words: ornithine decarboxylase, phosphoinositide 3-kinase, polyamine, Raf/MEK/ERK, Ras, translational regulation.

INTRODUCTION

The goal of the experiments described in the present study is to understand the mechanism by which ODC (ornithine decarboxylase) activity is induced during carcinogenesis by oncogenic *ras*. Ras is an essential component of receptor-mediated-signal-transduction pathways regulating growth and differentiation, and constitutively active Ras mutants have been implicated in many types of cancer (reviewed in [1]). ODC activity is dramatically induced in response to *ras* activation, and blocking ODC activity reverts both the transformed phenotype of *ras*-transformed cells [2–4] and carcinogenesis in several mouse models that acquire the mutated *ras* oncogene [5–9]. Recent results from this laboratory using rodent fibroblast models suggest strongly that ODC induction is both necessary for *ras* transformation and is mediated through more than one pathway downstream of Ras [4]. The Raf/MEK/ERK (where MEK stands for MAPK kinase and ERK for extracellular-signal-regulated kinase) cascade is the most extensively characterized of these pathways and is thought to be necessary for the transforming activity of oncogenic Ras [1]. However, depending on the cell type, additional effectors are now known to play important roles in Ras transformation.

The number of pathways activated by Ras include several signalling cascades in addition to Raf/MEK/ERK that are attractive candidates for ODC regulation. An example is the PI3K (phosphoinositide 3-kinase) pathway, which not only plays a negative regulatory role in apoptosis, but also controls translation through regulation of the translation initiation factor eIF4E

(eukaryotic initiation factor 4E) and its binding protein 4E-BP1, involving signalling by Akt/protein kinase B and mTOR (mammalian target of rapamycin) [10–13]. In addition, phosphorylation by ERK and p38 MAPK (mitogen-activated protein kinase) activates the eIF4E kinases Mnk1 and Mnk2, linking the pathways downstream of both Raf and MAP kinase kinase3/6 to the translational machinery [14].

Cap-dependent translational regulation of ODC has been well established and ODC activity is induced in eIF4E-overexpressing cells (4E-P2 cells) [15]. These cells exhibit characteristics of cellular transformation and down-regulation of ODC activity by either the irreversible inactivator α -difluoromethylornithine or expression of an ODC dominant-negative mutant abolishes the transformed phenotype [16,17]. Ras is also activated in 4E-P2 cells, as shown by an increased proportion of GTP-bound Ras, although no increase in the synthesis of the Ras protein was observed [15]. However, the pathways linking Ras activation and ODC induction remain to be determined. In the present study, we use several partial-loss-of-function Ras mutants as well as specific inhibitors to examine which pathways downstream of Ras are involved in ODC induction. The results establish a mechanism of ODC induction through Ras-activated signalling cascades, which involves activation of both ODC transcription and translation. In addition, the results suggest that at least two pathways downstream of Ras must be activated to produce high levels of ODC activity, with the Raf/MEK/ERK cascade and the PI3K cascade mediating transcription and translation of ODC respectively.

Abbreviations used: CAT, chloramphenicol acetyltransferase; eIF4E, eukaryotic initiation factor 4E; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; mTOR, mammalian target of rapamycin; ODC, ornithine decarboxylase; PI3K, phosphoinositide 3-kinase.

¹ e-mail lms17@psu.edu

EXPERIMENTAL

Plasmids

Plasmids coding for partial-loss-of-function mutants of H-Ras (pDCR-Ras12V, pDCR-Ras12V40C, pDCR-Ras12V35S and pDCR-Ras12V37G) were provided by Dr M. White (UT Southwestern, Dallas, TX, U.S.A.). The plasmids RSV-CAT (where RSV stands for Rous sarcoma virus and CAT for chloramphenicol acetyltransferase) and ODC-CAT were provided by Dr O. Jänne (University of Helsinki, Finland). The plasmid coding for the constitutively active Akt1 mutant (pUSE-myr-Akt) and the plasmid expressing a constitutively active MEK1 mutant (pUSE-MEK S218D/S222D) were purchased from Upstate Cell Signaling Solutions (Charlottesville, VA, U.S.A.). For co-transfection experiments of MEK and Akt, the MEK insert was removed by digesting with *NheI* and *XhoI* and ligated into the pcDNA3.1/Zeo(−) vector (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) digested with the same enzymes.

Cell culture

NIH-3T3 cells (a gift from Dr N. Sonenberg, McGill University, Montreal, Quebec, Canada) were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum, 4 mM glutamine and 100 µg/ml penicillin and streptomycin. Stable transfections of plasmids expressing Ras partial-loss-of-function mutants, Akt or MEK were performed using the LIPOFECTAMINE™ reagent (Invitrogen Life technologies) as described previously [4]. Stably transfected cells were maintained in 500 µg/ml Geneticin (Invitrogen Life Technologies). For co-transfection of Akt and MEK plasmids, cells were also maintained in 375 µg/ml zeocin (Invitrogen Life Technologies). For transcription experiments, Ras12V-overexpressing cells were transiently transfected with CAT reporter plasmids driven by either the RSV or the ODC promoter, as well as a β-gal plasmid, and CAT activity was measured 48 h later. CAT activity was compared with pDCR empty vector-expressing cells transiently transfected with the same plasmid. CAT activity was normalized to β-gal activity. Both CAT and β-gal activities were determined using ELISAs according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN, U.S.A.).

Biochemical assays

Cell extracts were prepared for enzyme assays as described previously and ODC was assayed in supernatant fractions by measuring the release of ¹⁴CO₂ from L-[1-¹⁴C]ornithine [16]. Intracellular polyamine content was measured using reversed-phase HPLC analysis and normalized to total cellular protein [18]. Protein assays were performed by the method of Bradford [19].

Western-blot analysis

Cells were harvested in a buffer containing 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin and 1 mM PMSF. For the analysis of Ras protein expression, the cell homogenate was resolved on a polyacrylamide gel and transferred to a PVDF membrane (Micron Separations, Westborough, MA, U.S.A.). The membrane was probed with a Ras monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and detected using a chemiluminescent detection system (Cell Signaling Technology, Beverly, MA, U.S.A.). For detection of phosphory-

lated ERK and Akt, rabbit polyclonal antibodies directed to both ERK1 and ERK2, or to Akt were used (Cell Signaling Technology) and total ERK1/ERK2 or Akt proteins were detected using rabbit polyclonal pan-ERK or Akt antibodies (Cell Signaling Technology). Total 4E-BP1 or phospho-4E-BP1 (Ser⁶⁵) were detected using rabbit polyclonal antibodies (Cell Signaling Technology). ODC protein was detected using an affinity-purified ODC polyclonal antibody [4]. Bands on Western-blot analysis were quantified using a Gene Genius Bioimaging System (Syngene, Cambridge, U.K.).

Determination of phosphorylated and unphosphorylated eIF4E

Cells were harvested as described earlier and the phosphorylated and unphosphorylated forms of eIF4E were separated by isoelectric focusing on a slab gel and quantified by protein immunoblot analysis using a monoclonal antibody against total eIF4E (a gift from Dr S. R. Kimball, Penn State University College of Medicine, Hershey, PA, U.S.A.) as described previously [20].

Northern-blot analysis

For Northern-blot analysis of ODC message, RNA was isolated from cells using the Totally RNA kit (Ambion, Austin, TX, U.S.A.) according to the manufacturer's instructions. Total RNA (20 µg) was separated on a 1.2 % (w/v) agarose-formaldehyde gel, transferred to nylon membrane (Amersham Biosciences) and UV-cross-linked using a Stratalinker (Stratagene, La Jolla, CA, U.S.A.). An antisense cRNA probe was synthesized from pGEM-ODC [21] using SP6 RNA polymerase and fluorescein-12-UTP (Roche Applied Science). Blots were hybridized overnight and analysed by fluorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Differences in RNA loading were normalized using ethidium bromide staining of rRNA.

Treatment of cells with inhibitors

NIH-3T3 cells stably expressing the Ras12V mutant were plated at a density of 50 000 cells/well in 24-well plates. After 24 h, the medium was changed and the signal-transduction inhibitors dissolved in DMSO were added. Inhibitors and concentrations were as follows: 25 µM PD98059, 10 µM LY294002, 200 nM rapamycin and 10 µM SB202190 (all were purchased from Calbiochem, San Diego, CA, U.S.A.). Control cells received an equal volume of DMSO. Cells were harvested 0, 6, 24, 48 and 72 h after the addition of inhibitors without changing the medium and then assayed for ODC activity. Results presented are the means of at least triplicate experiments. For the detection of phosphorylated proteins in inhibitor-treated cells, cells were serum-starved overnight and then treated with inhibitors for 90 min before harvesting for Western-blot analysis.

RESULTS

Expression of partial-loss-of-function Ras mutants

Point mutations in residues 32–40 of Ras are known to block the interaction of Ras with several of its target effectors [22,23]. These experiments made use of partial-loss-of-function Ras mutants to activate selectively the downstream pathways in NIH-3T3 cells and examine the contribution of each pathway to ODC induction. Since the activity of the ODC enzyme is known to be a good reflection of protein levels [24,25], ODC activity is used as a measure of ODC protein in these experiments. The Ras mutants

Table 1 ODC activities and Ras protein levels in NIH-3T3 cell clones with stable expression of Ras partial-loss-of-function mutants

Stable clones of NIH-3T3 cells expressing the Ras12V-activating mutant or the Ras partial-loss-of-function mutants Ras12V35S, Ras12V37G or Ras12V40C were plated and allowed to grow for 72 h without changing the medium, when they were harvested. Analysis of Ras protein by Western-blot analysis was performed on 20 or 100 μ g of total protein separated by SDS/PAGE (15% gel). Ras was detected using a monoclonal antibody. The results from two separate experiments using 20 μ g of protein are shown. The level of endogenous Ras protein in empty vector control cells was below the limit of detection. ODC activity obtained was compared with that in cells stably expressing the pDCR empty vector [empty vector control activity was 0.4 ± 0.1 nmol $\cdot$ (30 min) $^{-1}$ $\cdot$ (mg of protein) $^{-1}$, $N = 5$]. ODC activities are presented as a ratio of ODC in each Ras mutant to ODC in empty vector controls and are the means $\pm$ S.E.M. from 3–5 independent experiments.

Ras mutant (clone)	Ras protein (absorbance units)		Mutant/control ODC-fold increase (72 h)
	Experiment 1	Experiment 2	
Ras12V (12)	1.3	1.2	3.9 ± 1.0
Ras12V (17)	26.8	42.2	8.5 ± 2.0
Ras12V (15)	85.0	78.3	20.3 ± 3.6
Ras12V35S (14)	36.5	35.3	2.6 ± 0.3
Ras12V37G (2)	64.1	82.8	1.9 ± 0.5
Ras12V40C (1)	60.6	59.6	1.7 ± 0.3

used included Ras12V, which can bind to and activate pathways downstream of Raf, PI3K and Ral.GDS [22,23]; Ras12V40C, which does not activate Raf but retains its ability to activate PI3K and its downstream components [26,27]; Ras12V37G, which has been reported to interact selectively with Ral.GDS [26,28]; and Ras12V35S, which has been shown to bind to both Raf and Ral.GDS but not PI3K [26,29]. Since several Ras mutants activate multiple pathways, it is probable that overexpression of more than one of the mutant Ras proteins will result in increased ODC activity. However, the value of these mutants is to eliminate activation pathways.

The consequences of sustained Ras activation to ODC expression were determined by stable transfection of Ras partial-loss-of-function mutants, and isolation of at least 15 clones of each found to be resistant using G418 selection. There was a correlation between the amount of Ras protein expressed and the ODC activity in the Ras12V clones, with three representative clones shown in Table 1. In contrast, none of the Ras partial-loss-of-function mutants produced clones with more than approx. 3-fold increase in ODC activity. The clones with the highest Ras protein expression of each partial-loss-of-function mutant are shown (Table 1). Cells expressing either Ras12V37G or Ras12V40C were found to contain levels of Ras approximately equal to the highest expressing Ras12V clones (Ras12V-15). The highest expression of the Ras12V35S clones had approximately half the Ras protein level compared with the Ras12V-15 cells. The reason for this difference is not clear. However, Ras12V clone 12 and Ras12V35S clone 14 had approximately equal ODC activities, yet the Ras12V-12 cells expressed approx. 30-fold less Ras protein compared with the Ras12V35S-14 cells (Table 1), emphasizing the amplified effect of the activating mutant on ODC induction compared with the partial-loss-of-function mutant. Western-blot analysis of ODC protein confirmed that ODC levels were highest in Ras12V-transformed cells (results not shown). In addition, the activities of *S*-adenosylmethionine decarboxylase and spermidine/spermine *N*¹-acetyltransferase, other key enzymes of the polyamine pathway, were not changed in Ras12V cells compared with control cells at the time points tested (results not shown), suggesting that the response to *ras* transformation

Table 2 Polyamine levels in NIH-3T3 cells stably expressing Ras12V or pDCR empty vector

Cells stably expressing the indicated vectors were harvested after 72 h of growth, when the intracellular polyamine content was measured for each independent replicate plate using reversed-phase HPLC analysis and normalized to total cellular protein. Results are the means $\pm$ S.E.M.

Transfected plasmid	Polyamine measurements [nmol $\cdot$ (mg of protein) $^{-1}$]		
	Putrescine	Spermidine	Spermine
pDCR empty vector ($n = 9$)	< 0.1	16.0 ± 2.5	14.2 ± 2.8
pDCR-Ras12V ($n = 11$)	3.5 ± 0.2	26.2 ± 1.6	10.4 ± 0.4

in these cells is specific to ODC. Other studies using 10T1/2 cells showed an induction in both ODC and *S*-adenosylmethionine decarboxylase activities in response to Ras overexpression [30].

Polyamine levels were examined in Ras12V cells (clone 15) and compared with empty vector controls. Putrescine, a direct product of the ODC reaction, was below the limit of detection (approx. 0.1 nmol) in empty vector control cells. In contrast, putrescine levels were detected easily in Ras12V cells, reflecting the increased ODC activity (Table 2). Spermidine was also increased in Ras12V cells, but spermine levels were slightly lower. Therefore overexpression of ODC in response to Ras12V altered the distribution of the intracellular polyamines. The increased spermidine/spermine ratio seen in Ras12V cells is typical of active polyamine synthesis and cell proliferation. This change is reflected by a decreased doubling time in Ras12V-transformed cells of 25 h, as compared with a 33 h doubling time in parental control cells.

Isolation of specific pathways downstream of Ras

The results in Table 1 show that isolation of the Ral.GDS pathway using the Ras12V37G mutant did not increase ODC activity in the resulting cells examined. Additional stable clones were isolated expressing a constitutively active mutant of Akt (myr-Akt), thus activating elements downstream of the PI3K pathway. Since ODC is known to be regulated translationally in many systems, often resulting in large increases in enzyme activity with no change in mRNA levels, and Akt is part of a pathway known to control translation initiation (reviewed in [31]), it was of particular interest to determine the effects of activating translation factors downstream of Akt. Although several myr-Akt clones showed high levels of Akt activity, as assayed by 4E-BP1 phosphorylation, all had low levels of ODC activity and there was no correlation between Akt expression and ODC activity (results not shown). Eight separate clones were examined in both exponentially growing and confluent cultures, and none showed a sustained induction of ODC activity such as that seen in the Ras12V-transformed cells. Previous results using stable clones expressing v-Raf, thus activating the Raf/MEK/ERK pathway alone, demonstrated that activation of the Raf/MEK/ERK pathway is also not sufficient for complete induction of ODC activity by Ras [4]. These results were confirmed in the present study by transfecting a plasmid expressing the constitutively active MEK mutant S218D/S222D, which activates only the ERK pathway and eliminates the possibility of cross-talk between Raf and other pathways. This transfection also did not produce clones with ODC activity more than three times the control cells in ten separate clones tested (results not shown).

Figure 1 Phosphorylated ERK and 4E-BP1 levels in stable Akt/MEK clones with a range of ODC activities

(A) Five Akt/MEK-overexpressing clones were grown for 72 h, serum-starved for 24 h and then harvested. For each sample, 40 μ g of total protein was separated by SDS/PAGE on either a 10% (ERK1/ERK2) or a 15% (4E-BP1) gel. MEK and Akt activities were assayed by Western-blot analysis using phospho-specific antibodies for ERK1/ERK2 and 4E-BP1 respectively. Blots were stripped and re-blotted with antibodies specific for total ERK1/ERK2 and total 4E-BP1 to confirm equal loading of protein (results not shown). Quantification of the combined ERK1/ERK2 levels and the 4E-BP1 levels is shown below the gels. The results are representative of three independent experiments. Separate blots measuring total MEK and Akt in each clone confirmed overexpression of both proteins (results not shown). (B) Parallel plates were grown identically for ODC activity assay, but were not serum starved before harvest. The ODC activity in control cells was 0.11 nmol $\cdot$ (30 min) $^{-1}$ $\cdot$ (mg of protein) $^{-1}$.

The above results suggest that more than one pathway downstream of Ras controls the ODC response. To test whether co-ordinate activation of the Raf/MEK/ERK and PI3K pathways increases ODC activity, MEK S218D/S222D was co-transfected along with myr-Akt, and 20 separate clones were chosen and assayed for ODC activity. Figure 1(B) shows ODC activities in five of these clones. After 72 h of growth, expression of MEK and Akt together increased ODC activity by as much as 20-fold over control cells. Overexpression of MEK and Akt together resulted in up to six times more ODC activity when compared with the expression of Akt or MEK alone. Akt/MEK clones with a range of ODC levels were assayed for MEK and Akt activities by measuring phosphorylation of their products ERK and 4E-BP1 respectively (Figures 1A and 1B). Since four out of five clones tested had nearly equal levels of Akt activity, it was difficult to determine a correlation between Akt and ODC activities. Also, there is no perfect correlation between MEK and ODC activities, although the clones with the greatest ODC activities (clones 7 and 12), also have the highest levels of phosphorylated ERK1/ERK2. However, clones 3 and 19, which have similar phospho-ERK levels but very different levels of phospho-4E-BP1, have ODC activities that differ by 10-fold. These results suggest that the PI3K and the Raf/MEK/ERK pathways both contribute to ODC induction in Ras-transformed cells.

Response of ODC to kinase inhibitors

To extend the results obtained with the mutant Ras proteins, downstream effectors of Ras were selectively targeted using

Figure 2 Effect of kinase inhibitors on ODC activity in Ras12V-over-expressing cells

Ras12V-overexpressing cells were grown for 72 h in the presence of kinase inhibitors. The concentration of each inhibitor used was as follows: 25 μ M PD98059, 10 μ M LY294002, 200 nM rapamycin and 10 μ M SB202190. Control cells received DMSO vehicle. At the indicated time points between 0 and 72 h, cells were harvested without changing the medium and assayed for ODC activity. All points represent the means $\pm$ S.E.M. of at least triplicate experiments.

specific inhibitors of the following: MEK (25 μ M PD98059), PI3K (10 μ M LY294002), mTOR, which controls phosphorylation of proteins involved in translation initiation and is downstream of both PI3K and Akt (200 nM rapamycin), or p38MAPK (10 μ M SB202190), in cells stably expressing the Ras12V mutant and ODC activity was monitored over time. For these experiments as well as those that follow, the Ras12V clone with the highest ODC activity (clone 15) was used. In Ras12V-transformed cells treated with DMSO vehicle, ODC activity increased steadily as cells became confluent. This increase in ODC activity is typical of transformed cells, and is in contrast with non-transformed NIH-3T3 cells in which ODC activity peaks during exponential growth and then decreases to very low levels in confluent monolayers [25,32]. Inhibition of MEK, PI3K or mTOR resulted in decreases in ODC activity after 72 h (Figure 2). The most dramatic effect on ODC activity occurred in the presence of the MEK inhibitor PD98059, which resulted in virtually undetectable ODC activity after a 72 h exposure. In contrast, inhibition of the p38MAPK pathway actually induced ODC activity. After 72 h exposure to SB202190, ODC activity was almost twice that seen in DMSO-treated cells (Figure 2). In addition, both PD98059 and LY294002 reversed the transformed morphology of Ras12V-overexpressing cells over a 72 h exposure, whereas SB202190 and rapamycin did not (results not shown).

The specificity of the kinase inhibitors in this system was evaluated by exposing serum-starved Ras12V-transformed cells to either DMSO or to each inhibitor for 90 min. Cells were harvested and Raf/MEK/ERK pathway activation was measured by determining the levels of phosphorylated ERK1/ERK2, whereas PI3K pathway activity was measured by monitoring the levels of phosphorylated Akt/protein kinase B. The results are shown in Figure 3. Inhibition of either MEK or PI3K specifically inactivated their target pathways, with PD98059 treatment resulting in complete lack of ERK phosphorylation and LY294002 treatment almost completely inhibiting phosphorylation of Akt. In contrast, treatment of cells with the p38MAPK inhibitor SB202190 resulted in a compensatory increase in ERK1/ERK2 phosphorylation and also an inhibition of Akt phosphorylation. As expected, rapamycin, which inhibits mTOR, a downstream target of Akt, had no

Figure 3 Activation states of Raf/MEK/ERK and PI3K pathways in inhibitor-treated Ras12V cells

Ras12V-overexpressing cells were grown for 72 h, serum starved for 24 h and then treated with kinase inhibitors for 90 min at the concentrations indicated in Figure 2. The activation states of the Raf/MEK/ERK and PI3K pathways were measured by Western-blot analysis using phospho-specific antibodies for ERK1/ERK2 and Akt respectively. The blot was then stripped and re-blotted with antibodies specific for total ERK1/ERK2 and total Akt. Measurements were repeated at least three times.

effect on the phosphorylation state of ERK1/ERK2 or Akt (results not shown).

Transcriptional and translational regulation of ODC in the presence of kinase inhibitors

Since it is known that ODC is regulated at both the transcription and translation levels (reviewed in [31]), the contributions of each pathway to changes in ODC mRNA and protein were also assessed. Northern-blot analysis of Ras12V-transformed cells grown for 72 h showed that steady-state levels of ODC mRNA were increased 3.9-fold higher than the levels of NIH-3T3 cells transfected with empty vector, considerably less than the 20-fold increase in ODC activity brought about by expression of the Ras12V mutant (Table 1). Inhibition of MEK in these cells using 25 μ M PD98059 decreased the level of ODC mRNA almost to empty vector control level. This is consistent with the dramatic inhibition of ODC activity seen over the same time period (Figure 2). Although the inhibition of the PI3K pathway with either 10 μ M LY294002 or 200 nM rapamycin decreased substantially the ODC activity (Figure 2), both inhibitors had no effect on ODC mRNA levels.

To determine directly the ability of activated Ras to stimulate transcription from the ODC promoter, Ras12V-transformed cells or empty vector control NIH-3T3 cells were transiently transfected with a CAT reporter gene driven by the entire ODC promoter of 1.6 kb. As expected, transcription from the ODC promoter was higher in Ras12V-transformed cells compared with empty vector controls, but only approx. 2-fold ($P = 0.001$), again suggesting that post-transcriptional mechanisms must account for much of the ODC induction seen in NIH-3T3 cells transformed

Figure 4 Phosphorylation states of eIF4E and 4E-BP1 in inhibitor-treated Ras12V cells

Ras12V-overexpressing cells were grown for 72 h, serum starved for 24 h and then treated with kinase inhibitors for 90 min at the concentrations indicated in Figure 2. Cells were harvested and proteins separated on an isoelectric focusing slab gel for detection of eIF4E (A), or a 15% (w/v) polyacrylamide gel for detection of 4E-BP1 (B). Western-blot analysis was performed using antibodies directed against either total eIF4E (A) or total 4E-BP1 (B). Similar results were obtained in separate experiments with Western blots using antibodies specific for the phosphorylated forms of eIF4E and 4E-BP1 (results not shown).

by Ras. To confirm the effect of MEK inhibition on ODC transcription, cells were treated with the MEK inhibitor PD98059 (25 μ M) added to the medium 5 h after the start of transfection and CAT levels were measured after 48 h. Transcription from the ODC promoter was decreased by MEK inhibition in Ras12V cells to levels not significantly different from those seen in empty vector control cells in the absence of the inhibitor. On the other hand, inhibition of MEK in empty vector control cells did not significantly change the ODC transcription. The MEK inhibitor also had no effect on CAT expression from an RSV-CAT control plasmid.

The transcription results suggest that the primary regulation of ODC activity in Ras-transformed cells is at the level of translation. However, another possibility that would explain the reduction in ODC activity in the presence of kinase inhibitors is a decrease in stability of the ODC protein. ODC is a very short-lived protein *in vivo*, with a half-life of 30–90 min depending on the cell type. Based on the loss of activity in the presence of cycloheximide, the half-life of ODC in NIH-3T3 control cells was found to be 66 min and this was increased in Ras12V-transformed cells to approx. 90 min in two separate experiments. When cells were preincubated with either 25 μ M PD98059 or 10 μ M LY294002 before the addition of cycloheximide, ODC half-life was decreased to 60 min. However, this increase in the rate of degradation back to control levels cannot in itself account for the profound loss in ODC activity, which occurs in the presence of these inhibitors.

Phosphorylation levels of translation-initiation factor eIF4E and its binding protein 4E-BP1, which regulate cap-dependent translation and may be involved in the translational regulation of ODC (reviewed in [31]), were also examined in the presence of kinase inhibitors. In Ras12V-transformed cells, 10% of intracellular eIF4E is present in phosphorylated form, and this phosphorylation was decreased to undetectable levels after inhibition of either MEK or PI3K (Figure 4A). Availability of eIF4E in the cell is regulated by its binding to 4E-BP1. In its non-phosphorylated state, 4E-BP1 binds to eIF4E, inhibiting cap-dependent translation. Hyperphosphorylation of 4E-BP1 disrupts this

binding, activating cap-dependent translation (reviewed in [33]). The γ -form represents the hyperphosphorylated form of 4E-BP1 and is the only form that can release eIF4E [34]. Inhibition of PI3K in Ras12V-transformed cells resulted in the reduction of this γ -phosphorylated form (Figure 4B). Interestingly, inhibition of MEK, which decreased ODC activity almost to control levels, did not affect the levels of the γ -form of 4E-BP1 in these cells (Figure 4B). MEK inhibition also did not change the phosphorylation state of Ser⁶⁵, as shown using an antibody specific for phosphorylated Ser⁶⁵ of the mouse 4E-BP1 sequence (results not shown). This serine residue is thought to be the major ERK phosphorylation site of 4E-BP1 [35].

DISCUSSION

ODC activity is dramatically induced in response to *ras* activation, and previous studies have demonstrated that induction of ODC activity is necessary for the transformation of NIH-3T3 cells by oncogenic *ras* [4]. In the present study, the mechanism by which ODC activity is increased was investigated, with the goal of understanding which Ras-dependent pathways are responsible. The results obtained by using Ras partial-loss-of-function mutants, constitutively active forms of several downstream effectors, as well as kinase inhibitors, indicate that more than one pathway downstream of Ras is required for the complete induction of ODC activity. Also, activation of the Raf/MEK/ERK and PI3K pathways contribute to transcriptional and translational up-regulation of ODC in Ras-transformed cells.

To assess the ability of known Ras-effector pathways to induce ODC in NIH-3T3 cells, stable clones expressing Ras partial-loss-of-function mutant proteins were evaluated for ODC activities. Clones overexpressing Ras12V, which activates all Ras-effector pathways, induced ODC activity up to 20-fold more than the empty vector control levels. These ODC activities are similar to those produced in previous experiments overexpressing the Ras61L-activating mutant [4]. Ras12V35S, which can bind both the Raf and Ral.GDS, was the only partial-loss-of-function mutant capable of inducing ODC activity consistently above that seen in empty vector controls. This is probably owing to the Raf-dependent pathways, since activation of factors downstream of RalGEFs, such as JNK [36], has been associated with ODC inhibition and polyamine depletion [37], making it less probable that activation of Ral.GDS contributes to ODC induction. This is also supported by the results obtained with stable clones overexpressing Ras12V37G, many of which had ODC activities below even empty vector control cells (Table 1 and results not shown).

Previous studies showed that activation of the Raf/MEK/ERK pathway using an activated form of Raf resulted in only a modest ODC induction in NIH-3T3 cells [4], suggesting that up-regulation of this pathway alone is also not sufficient to have a considerable effect on ODC expression. Expression of a constitutively active MEK in the present study confirmed these results. Stable expression of either Ras12V40C, which activates PI3K or of constitutively active Akt, also did not increase ODC activity in the resulting clones. However, when activated forms of Akt and MEK were expressed simultaneously, ODC induction was comparable with that seen in Ras12V cells. Phosphorylation assays of downstream products ERK1/ERK2 and 4E-BP1 indicated that both pathways were highly active in Akt/MEK clones with high ODC activity.

To confirm co-operation of Ras-effector pathways in ODC induction, cells stably expressing the Ras12V activating mutant were treated with inhibitors of kinases known to act downstream of Ras. Inhibition of MEK, PI3K or mTOR all resulted in a decrease in ODC activity in Ras12V cells.

Therefore these results, combined with the Ras effector results, strongly suggest that the up-regulation of ODC in Ras-transformed cells is linked to the Raf/MEK/ERK and PI3K pathways. Co-ordinate activation of the PI3K and Raf/MEK/ERK pathways to induce ODC activity has also been noted in a variant of L1210 cells that are resistant to α -difluoromethylornithine and contain an amplified ODC gene (DR cells) [38,39]. In those experiments, the change in ODC activity was examined in response to serum stimulation in the presence of inhibitors of MEK or PI3K. However, the activation states of Ras and its effectors are unknown in DR cells.

In contrast with the results seen in the presence of MEK and PI3K inhibition, treatment of cells with the p38MAPK inhibitor SB202190 resulted in an increase in ODC activity. This effect was reproducible in several experiments. Whereas the mechanism is unclear, a compensatory increase in ERK1/ERK2 phosphorylation was seen in the presence of this inhibitor. Therefore the increase in ODC activity seen in the presence of p38MAPK inhibition may be the result of compensatory ERK activation. These results are in agreement with those obtained recently in studies examining induction of ODC in response to histamine and PMA in human ECV304 cells, in which treatment with either SB202190 or another p38MAPK inhibitor, SB203580, also resulted in increased ERK phosphorylation and increased ODC activity [40]. Several other recent reports in various systems have shown increases in ERK1/ERK2 activity in response to inhibition of p38 MAPK (see [41] and references therein). Future experiments will examine this possible interplay between the p38MAPK and ERK pathways in the regulation of ODC activity.

To determine the mechanism by which ODC activity is induced in response to Ras transformation, ODC mRNA levels were evaluated in cells overexpressing Ras12V in the presence of MEK and PI3K inhibitors. The amount of ODC mRNA is increased <4-fold in Ras12V-transformed cells, and is completely dependent on the Raf/MEK/ERK activation, since it is abolished by inhibition of MEK. The results suggest that this small increase in transcription may be necessary for the complete induction of ODC, since MEK inhibition also completely blocks the increase in ODC activity in Ras12V cells. The increase in ODC activity in Ras12V cells, is decreased substantially but not completely by PI3K inhibition, while having no effect on the level of ODC mRNA. Therefore the mechanism of ODC regulation in Ras12V-overexpressing cells appears to involve both new transcription and increased translation of mRNA into active protein.

The translation of ODC mRNA is known to be negatively regulated by changes in the intracellular polyamines spermidine and spermine (reviewed in [31]). Thus, when polyamine levels are low, the synthesis of ODC is increased, and an increase in polyamine content causes a corresponding decrease in ODC protein synthesis. Previous experiments have suggested that an increase in the levels of eIF4E may allow for the synthesis of ODC even in the presence of high polyamine levels, which would repress ODC translation in cells with lower levels of the initiation factor [16,17]. This may be the case in transformed cells, which generally contain higher polyamine levels compared with normal cells, yet also maintain high levels of ODC activity. Experiments in AR4-2J pancreatic tumour cells, which show increased phosphorylation of 4E-BP1 and high levels of eIF4E compared with normal cells, also showed increased translational initiation of ODC mRNA [42]. Enhancement of RNA helicase activity and increased eIF4E phosphorylation have been reported in mouse FM3A cells, which have very high levels of ODC protein [43]. Additional evidence that the intracellular availability of active eIF4E correlates with ODC translation has been provided

in studies using IEC-6 intestinal epithelial cells, where it has been shown that rapamycin, which blocks phosphorylation of 4E-BP1, inhibits the induction of ODC in response to serum [44]. Increased ODC activity was also correlated with the availability of free eIF4E in leucine-stimulated L6 myoblasts [45].

As shown in the Results section, the total amount of eIF4E protein is not appreciably different in untreated Ras12V-transformed NIH-3T3 cells compared with cells treated with inhibitors of either MEK or PI3K. However, phosphorylation of eIF4E is decreased to undetectable levels in Ras12V cells in response to inhibition of either PI3K or MEK. Hyperphosphorylation of its binding protein 4E-BP1 is decreased only in the presence of PI3K inhibition, with inhibition of MEK having no effect. Since inhibitors of either MEK or PI3K dramatically decrease ODC synthesis in Ras12V-transformed cells, this suggests that, in addition to the amount of hyperphosphorylated 4E-BP1, the amount of phosphorylated eIF4E available is also important for an efficient ODC translation in this system. Interestingly, exposure of Ras12V cells to the p38 MAPK inhibitor SB202190, which as mentioned earlier increases the ODC activity and ERK1/ERK2 phosphorylation in these cells, also increased the phosphorylation of eIF4E from 10 % of total 4E protein to 30 % (L.M. Shantz and S.R. Kimball, unpublished work).

The eIF4E kinases Mnk1 and Mnk2 are known targets of both the Raf/MEK/ERK and p38 MAPK pathways [14,46,47], and the role of eIF4E phosphorylation in translation initiation has been increasingly studied in recent years. Results using transient transfection of activated Mnk1 have suggested that the increased phosphorylation of eIF4E is not sufficient to affect general protein synthesis [48]. However, this does not rule out the possibility of a subset of proteins being regulated by eIF4E phosphorylation. Indeed, previous studies have shown that overexpression of eIF4E in NIH-3T3 cells induces ODC activity to levels comparable with those seen in the current studies for Ras12V cells [16], although the phosphorylation levels of eIF4E in these cells were not investigated. Recent findings also suggest the possibility that phosphorylation of eIF4E may be more important for the translation of newly synthesized RNAs [49], which would include those increased during mitogenic stimulation, such as ODC. This is in agreement with the results of the present study for ODC in Ras12V-transformed cells, which suggest that activation of the Raf/MEK/ERK pathway leads to increased transcription of ODC message, and translation is then regulated by activation of the cap-dependent translational machinery through the PI3K and the Raf/MEK/ERK pathways.

In summary, the experiments described here examined in detail the signalling pathways involved in the ODC response to Ras. Results show for the first time that both the Raf/MEK/ERK and PI3K pathways are needed for induction of ODC activity. Activation of these pathways increases levels of both ODC RNA and protein, resulting in substantial up-regulation in ODC activity that accompanies Ras transformation.

This research was supported by grant no. CA82768 from the National Institutes of Health and a Penn State College of Medicine Cancer Research Grant. The generous contribution of plasmids from Dr M. White and Dr O. Jänne and help with the eIF4E isoelectric focusing gel and Western-blot analysis from Dr S.R. Kimball are gratefully acknowledged. The excellent technical assistance of Suzanne Sass-Kuhn is also gratefully acknowledged.

REFERENCES

- Khosravi-Far, R. and Der, C. J. (1994) The Ras signal transduction pathway. *Cancer Metastasis Rev.* **13**, 67–89
- Auvinen, M., Paasinen, A., Andersson, L. A. and Hölttä, E. (1992) Ornithine decarboxylase activity is critical for cell transformation. *Nature (London)* **360**, 355–358
- Auvinen, M. (1997) Cell transformation, invasion, and angiogenesis: a regulatory role for ornithine decarboxylase and polyamines. *J. Natl. Cancer Inst.* **89**, 533–537
- Shantz, L. M. and Pegg, A. E. (1998) Ornithine decarboxylase induction in transformation by H-Ras and RhoA. *Cancer Res.* **58**, 2748–2753
- Megosh, L., Gilmour, S. K., Rosson, D., Peralta Soler, A., Blessing, M., Sawicki, J. A. and O'Brien, T. G. (1995) Increased frequency of spontaneous skin tumors in transgenic mice which overexpresses ornithine decarboxylase. *Cancer Res.* **55**, 4205–4209
- Peralta Soler, A., Gilliard, G., Megosh, L., George, K. and O'Brien, T. G. (1998) Polyamines regulate expression of the neoplastic phenotype in mouse skin. *Cancer Res.* **58**, 1654–1659
- Lan, L., Trempus, C. and Gilmour, S. K. (2000) Inhibition of ornithine decarboxylase (ODC) decreases tumor vascularization and reverses spontaneous tumors in ODC/Ras transgenic mice. *Cancer Res.* **60**, 5696–5703
- Feith, D. J., Shantz, L. M. and Pegg, A. E. (2001) Targeted antizyme expression in the skin of transgenic mice reduces tumor promoter induction of ornithine decarboxylase and decreases sensitivity to chemical carcinogenesis. *Cancer Res.* **61**, 6073–6081
- Gupta, S., Ahmad, N., Marengo, S. R., MacLennan, G. T., Greenberg, N. M. and Mukhtar, H. (2000) Chemoprevention of prostate carcinogenesis by α -difluoromethylornithine in TRAMP mice. *Cancer Res.* **60**, 5125–5133
- Gingras, A.-C., Kennedy, S. G., O'Leary, M. A., Sonenberg, N. and Hay, N. (1998) 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev.* **12**, 502–513
- Gille, H. and Downward, J. (1999) Multiple Ras effector pathways contribute to G1 cell cycle progression. *J. Biol. Chem.* **274**, 22033–22040
- Polunovsky, V. A., Gingras, A.-C., Sonenberg, N., Peterson, M., Tan, A., Rubins, J. B., Manivel, J. C. and Bitterman, P. B. (2000) Translational control of the antiapoptotic function of Ras. *J. Biol. Chem.* **275**, 24776–24780
- Sheng, H., Shao, J. and DuBois, R. N. (2001) Akt/PKB activity is required for Ha-Ras-mediated transformation of intestinal epithelial cells. *J. Biol. Chem.* **276**, 14498–14504
- Wang, X., Flynn, A., Waskiewicz, A. J., Webb, B. L. J., Vries, R. G., Baines, I. A., Cooper, J. A. and Proud, C. G. (1998) The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. *J. Biol. Chem.* **273**, 9373–9377
- Lazaris-Karatzas, A., Smith, M. R., Fredrickson, R. M., Jaramillo, M. L., Liu, Y., Kung, H. and Sonenberg, N. (1992) Ras mediates translation initiation factor 4E-induced malignant transformation. *Genes Dev.* **6**, 1631–1642
- Shantz, L. M. and Pegg, A. E. (1994) Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. *Cancer Res.* **54**, 2313–2316
- Shantz, L. M., Hu, R.-H. and Pegg, A. E. (1996) Regulation of ornithine decarboxylase in a transformed cell line that overexpresses translation initiation factor eIF-4E. *Cancer Res.* **56**, 3265–3269
- Shantz, L. M., Holm, I., Jänne, O. A. and Pegg, A. E. (1992) Regulation of S-adenosylmethionine decarboxylase activity by alterations in the intracellular polyamine content. *Biochem. J.* **288**, 511–518
- Bradford, M. M. (1976) A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72**, 248–254
- Kimball, S. R., Jurasinski, C. V., Lawrence, Jr, J. C. and Jefferson, L. S. (1997) Insulin stimulates protein synthesis in skeletal muscle by enhancing the association of eIF-4E and eIF-4G. *Am. J. Physiol.* **272**, C754–C759
- Lu, L., Stanley, B. A. and Pegg, A. E. (1991) Identification of residues in ornithine decarboxylase essential for enzymatic activity and for rapid protein turnover. *Biochem. J.* **277**, 671–675
- White, M. A., Nicolette, C., Minden, A., Polverino, A., Van Aelst, L., Karin, M. and Wigler, M. H. (1995) Multiple Ras functions can contribute to mammalian cell transformation. *Cell (Cambridge, Mass.)* **80**, 533–541
- Joneson, T., White, M. A., Wigler, M. H. and Bar-Sagi, D. (1996) Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. *Science* **271**, 810–812
- Kahana, C. and Nathans, D. (1985) Translational regulation of mammalian ornithine decarboxylase by polyamines. *J. Biol. Chem.* **260**, 15390–15393
- Hölttä, E., Sistonen, L. and Alitalo, K. (1988) The mechanisms of ornithine decarboxylase deregulation in c-Ha-ras oncogene-transformed NIH-3T3 cells. *J. Biol. Chem.* **263**, 4500–4507
- Rodríguez-Viciano, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A. and Downward, J. (1997) Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by ras. *Cell (Cambridge, Mass.)* **89**, 457–467

- 27 Khwaja, A., Rodriguez-Vician, P., Wennström, S., Warne, P. H. and Downward, J. (1997) Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J.* **16**, 2783–2793
- 28 Fuller, S. J., Finn, S. G., Downward, J. and Sugden, P. H. (1998) Stimulation of gene expression in neonatal rat ventricular myocytes by Ras is mediated by Ral guanine nucleotide dissociation stimulator (RalGDS) and phosphatidylinositol 3-kinase in addition to Raf. *Biochem. J.* **335**, 241–246
- 29 Khosravi-Far, R., White, M. A., Westwick, J. K., Soltski, P. A., Chrzanoska-Wodnicka, M., Van Aelst, L., Wigler, M. H. and Der, C. J. (1996) Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathways is sufficient to cause tumorigenic transformation. *Mol. Cell. Biol.* **16**, 3923–3933
- 30 Hurta, R. A. R. (2000) Altered ornithine decarboxylase and S-adenosylmethionine decarboxylase expression and regulation in mouse fibroblasts transformed with oncogenes or constitutively active mitogen-activated protein (MAP) kinase kinase. *Mol. Cell. Biochem.* **215**, 81–92
- 31 Shantz, L. M. and Pegg, A. E. (1999) Translational regulation of ornithine decarboxylase and other enzymes of the polyamine pathway. *Int. J. Biochem. Cell Biol.* **31**, 107–122
- 32 Heby, O. (1981) Role of polyamines in the control of cell proliferation and differentiation. *Differentiation* **19**, 1–20
- 33 Raught, B. and Gingras, A.-C. (1999) eIF4E activity is regulated at multiple levels. *Int. J. Biochem. Cell Biol.* **31**, 43–57
- 34 Haghighat, A. and Sonenberg, N. (1997) eIF4G dramatically enhances the binding of eIF4E to the mRNA 5'-cap structure. *J. Biol. Chem.* **272**, 21677–21680
- 35 Herbert, T. P., Tee, A. R. and Proud, C. G. (2002) The extracellular signal-regulated kinase pathway regulates the phosphorylation of 4E-BP1 at multiple sites. *J. Biol. Chem.* **277**, 11591–11596
- 36 DeRuiter, N. D., Wolthuis, R. M. F., Van Dam, H., Burgering, B. M. T. and Bos, J. L. (2000) Ras-dependent regulation of c-Jun phosphorylation is mediated by the Ral guanine nucleotide exchange factor-Ral pathway. *Mol. Cell. Biol.* **20**, 8480–8488
- 37 Ray, R. M., McCormack, S. A. and Johnson, L. R. (2001) Polyamine depletion arrests growth of IEC-6 and Caco-2 cells by different mechanisms. *Am. J. Gastrointest. Liver Physiol.* **281**, G37–G43
- 38 Flamigni, F., Marmiroli, S., Capanni, C., Stefanelli, C., Guarnieri, C. and Caldarera, C. M. (1997) Phosphatidylinositol 3-kinase is required for the induction of ornithine decarboxylase in leukemia cells stimulated to growth. *Biochem. Biophys. Res. Commun.* **239**, 729–733
- 39 Flamigni, F., Facchini, A., Capanni, C., Stefanelli, C., Tantini, B. and Caldarera, C. M. (1999) p44/42 mitogen-activated protein kinase is involved in the expression of ornithine decarboxylase in leukaemia L1210 cells. *Biochem. J.* **341**, 363–369
- 40 Flamigni, F., Facchini, A., Giordano, E., Tantini, B. and Stefanelli, C. (2001) Signaling pathways leading to the induction of ornithine decarboxylase: opposite effects of p44/42 mitogen-activated protein kinase (MAPK) and p38 MAPK inhibitors. *Biochem. Pharmacol.* **61**, 25–32
- 41 Keeton, A. B., Amsler, M. O., Venable, D. Y. and Messina, J. L. (2002) Insulin signal transduction pathways and insulin-induced gene expression. *J. Biol. Chem.* **277**, 48565–48573
- 42 Pyronnet, S., Gingras, A.-C., Bouisson, M., Kowalski-Chauvel, A., Seva, C., Vaysse, N., Sonenberg, N. and Pradayrol, L. (1998) Gastrin induces phosphorylation of eIF4E binding protein 1 and translation initiation of ornithine decarboxylase mRNA. *Oncogene* **16**, 2219–2227
- 43 Shimogori, T., Suzuki, T., Kashiwagi, K., Kakinuma, Y. and Igarashi, K. (1996) Enhancement of helicase activity and increase of eIF-4E phosphorylation in ornithine decarboxylase-overproducing cells. *Biochem. Biophys. Res. Commun.* **222**, 748–752
- 44 Seidel, E. R. and Ragan, V. L. (1997) Inhibition by rapamycin of ornithine decarboxylase and epithelial cell proliferation in intestinal IEC-6 cells in culture. *Br. J. Pharmacol.* **120**, 571–574
- 45 Kimball, S. R., Shantz, L. M., Horetsky, R. L. and Jefferson, L. S. (1999) Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6. *J. Biol. Chem.* **274**, 11647–11652
- 46 Waskiewicz, A. J., Johnson, J. C., Penn, B., Mahalingam, M., Kimball, S. R. and Cooper, J. A. (1999) Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 *in vivo*. *Mol. Cell. Biol.* **19**, 1871–1880
- 47 Scheper, G. C., Morrice, N. A., Kleijn, M. and Proud, C. G. (2001) The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells. *Mol. Cell. Biol.* **21**, 743–754
- 48 Herbert, T. P., Kilhams, G. R., Batty, I. H. and Proud, C. G. (2000) Distinct signalling pathways mediate insulin and phorbol ester-stimulated eukaryotic initiation factor 4F assembly and protein synthesis in HEK-293 cells. *J. Biol. Chem.* **275**, 11249–11256
- 49 Scheper, G. C., van Kollenburg, B., Hu, J., Luo, Y., Goss, D. J. and Proud, C. G. (2002) Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. *J. Biol. Chem.* **277**, 3303–3309

Received 27 May 2003/29 September 2003; accepted 30 September 2003

Published as BJ Immediate Publication 30 September 2003, DOI 10.1042/BJ20030778