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Ras Transformation of RIE-1 Cells Activates Cap-Independent Translation of Ornithine Decarboxylase: Regulation by the Raf/MEK/ERK and Phosphatidylinositol 3-Kinase Pathways

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Ras Transformation of RIE-1 Cells Activates Cap-Independent Translation of Ornithine Decarboxylase: Regulation by the Raf/MEK/ERK and Phosphatidylinositol 3-Kinase Pathways

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Abstract

Ornithine decarboxylase (ODC) is the first and generally rate-limiting enzyme in polyamine biosynthesis. Deregulation of ODC is critical for oncogenic growth, and ODC is a target of *Ras*. These experiments examine translational regulation of ODC in RIE-1 cells, comparing untransformed cells with those transformed by an activated Ras12V mutant. Analysis of the ODC 5′ untranslated region (5′UTR) revealed four splice variants with the presence or absence of two intronic sequences. All four 5′UTR species were found in both cell lines; however, variants containing intronic sequences were more abundant in Ras-transformed cells. All splice variants support internal ribosome entry site (IRES)–mediated translation, and IRES activity is markedly elevated in cells transformed by Ras. Inhibition of Ras effector targets indicated that the ODC IRES element is regulated by the phosphorylation status of the translation factor eIF4E. Dephosphorylation of eIF4E by inhibition of mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK) or the eIF4E kinase Mnk1/2 increases ODC IRES activity in both cell lines. When both the Raf/MEK/ERK and phosphatidylinositol 3-kinase/mammalian target of rapamycin pathways are inhibited in normal cells, ODC IRES activity is very low and cells arrest in G_1. When these pathways are inhibited in Ras-transformed cells, cell cycle arrest does not occur and ODC IRES activity increases, helping to maintain high ODC activity.

Introduction

Ornithine decarboxylase (ODC) has gained recognition as a critical target for tumorigenesis, especially for cancers triggered by oncogenes such as *Myc* and *Ras* (**1**, **2**). Curtailing ODC activity is sufficient to revert the transformation process *in vitro* and to inhibit the genesis of tumors, making it an ideal target for treatment and prevention of certain types of cancers (reviewed in ref. **3**). The primary function of ODC is to regulate the intracellular levels of the growth-essential polyamines by catalyzing the synthesis of the diamine putrescine from its precursor ornithine (reviewed in ref. **4**). Our goal in this study is to understand the intricacies of ODC regulation in response to Ras transformation. *Ras* is one of the most commonly mutated oncogenes in human cancers, and previous studies have shown that ODC is a target of Ras (**5**, **6**). Most *in vitro* studies characterizing ODC regulation in response to Ras have been carried out using fibroblasts (**7**– **9**). However, studies using RIE-1 cells have highlighted several differences between epithelial cells and fibroblasts with respect to the Ras effector pathways necessary for the transformation process (**10**). The observation of such differences is significant, as the majority of Ras-activated cancers are epithelial in origin. To understand the regulation of ODC in a more relevant model, the studies described here were carried out using Ras-transformed RIE-1 cells and their parental controls.

Ras activation in response to growth factors or constitutively active Ras mutants induces ODC activity by a complex mechanism involving transcription, translation, and changes in protein stability (**8**, **11**, **12**). Previous studies suggest that ODC synthesis is regulated predominantly by translation, especially at the level of initiation (**13**, **14**). One of the early steps in translation initiation is the recognition of the m^7GTP cap by the rate-limiting translation initiation factor eIF4E, which is part of the eIF4F cap-binding complex (**15**, **16**). Activation of Ras facilitates enhanced translation initiation by activating phosphatidylinositol 3-kinase (PI3K), which, in turn, activates Akt and mammalian target of rapamycin (mTOR) to facilitate the phosphorylation of the eIF4E binding protein 4EBP1 (**17**, **18**). Hypophosphorylated 4EBP1 interferes with eIF4F complex formation and inhibits translation initiation (**17**, **18**). Additionally, phosphorylation of eIF4E itself can be regulated by Ras through activation of the Mnk1/2 kinases, which are controlled by the Raf/mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK and p38 MAP kinase (MAPK) pathways (**19**). Although the role of eIF4E phosphorylation in regulating translation has yet to be defined, recent studies suggest that phosphorylation may play a significant role in the transformation properties of eIF4E (**20**).

Although translation via the cap is the predominant mode of ODC translation, under specific conditions such as the G_2-M phase of the cell cycle or during apoptosis, ODC can be translated in a cap-independent manner (**21**, **22**). Cap-independent translation is well-documented for viral RNAs that lack a cap structure and are translated efficiently by recruiting ribosomes directly onto regions within their 5′ untranslated region (5′UTR) termed internal ribosome entry sites (IRES; ref. **23**). IRES-mediated translation has been reported for other cellular RNAs important for cell growth, including c-Myc and cyclin D1 (**24**). ODC exhibits a biphasic induction of activity, once at the G_1-S boundary and again at the G_2-M phase of the cell cycle (**25**). ODC activity is increased during G_2-M in spite of an overall inhibition of global protein synthesis, and ODC activity during this phase is not responsive to the effects of the mTOR inhibitor rapamycin (**21**). These results suggest that ODC activity during G_2-M is regulated in a cap-independent manner.

Our studies analyze the effects of oncogenic Ras on ODC synthesis by cap-independent translational mechanisms. Ras activation enhanced IRES-mediated translational activity of ODC, which is regulated by both the Raf/MEK/ERK and PI3K pathways. Although the PI3K pathway regulates ODC IRES–mediated translation indirectly by altering cell cycle progression, ODC IRES activity can be regulated independently of cell cycle changes through the Raf/MEK/ERK pathway. Moreover, such regulation hinges upon the phosphorylation status of eIF4E.

Materials and Methods

Cell culture.

RIE-1 cells (a kind gift of Dr. K. Brown, University of Cambridge, Cambridge, United Kingdom) and 4E-P2 cells (**26**) were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 μg/mL penicillin and streptomycin. RIE-1 cells were stably transfected with Ras12V (**11**) or empty vector using LipofectAMINE plus reagent (Invitrogen). Stably transfected cells were selected in 400 μg/mL geneticin (G418).

Screening for ODC 5′UTR splice variants.

Total RNA was isolated using RNaqueous-4PCR kit (Ambion) and cDNA was synthesized from 2 μg RNA using the GC-rich SuperScript First-Strand cDNA synthesis kit (Invitrogen). Four percent of the cDNA synthesized was used in subsequent PCR amplification reactions. The ODC 5′UTR was cloned into the bicistronic plasmid pRΔEF with *Renilla* and Firefly luciferase reporters (a kind gift of Dr. P. Sarnow, Stanford University, Stanford, CA). The entire ODC 5′UTR was amplified from cDNA using forward and reverse primers introducing *Eco*RI overhangs into the 5′ end and 3′ end of the ODC 5′UTR. Both the empty vector and the amplified ODC 5′UTR were then digested with *Eco*RI, ligated together, and transformed into competent bacterial cells. Approximately 20 bacterial clones for both Ras12V and control cells were sequenced with a reverse primer Fluc01 (5′-GGCGCCGGGCCTTTCTTTATG-3′) complementary to the 5′ end of Firefly luciferase to identify the presence of ODC 5′UTR in sense or antisense orientations. Sequencing also revealed the presence of four 5′UTR splice variants of 273, 286, 290, and 303 nucleotides in length.

Distribution of 5′UTR variants.

ODC 5′UTR cDNA was PCR amplified and the PCR samples were analyzed by capillary electrophoresis using the HDA-GT12 capillary electrophoresis instrument (Transgenomics, Inc.). A 2 kb DNA mass standard was mixed with each PCR sample and analyzed in the same capillary to determine the concentration of each PCR product. Normalized area under each peak was measured using BIOCAL software (Transgenomics). DNA concentration for each peak was determined using the normalized area under the 2 kb DNA standard. Total normalized area under each region was added together to determine DNA concentration for each expected PCR product.

Dual luciferase assay and cell cycle analysis.

For dual luciferase assays, RIE-1 cells, Ras12V cells, or 4E-P2 cells were transfected with ODC bicistronic plasmids cloned into the pcDNA3.0 backbone (Invitrogen). Ras12V cells and RIE-1 cells were also transfected with pRMF (a generous gift of Dr. Anne Willis, University of Nottingham, United Kingdom), a bicistronic plasmid containing the c-Myc IRES. Twenty-four hours following transfection, cells were extracted in lysis buffer and assayed using the Dual-luciferase kit (Promega). For inhibitor treatments, 6 h after transfection, the medium was replaced with DMEM supplemented with 10% FBS and treated with either the vehicle DMSO or 100 nmol/L rapamycin (Calbiochem), 20 μmol/L LY294002 (Tocris Cookson), 50 μmol/L PD98059 (Calbiochem), or 20 μmol/L CGP57380 (Calbiochem). After 24 h, cells were extracted as described above for luciferase assays or fixed in 70% ethanol, washed with PBS, and stained with propidium iodide (Sigma) for flow cytometric analysis. Percentage of cells in each phase of the cell cycle was estimated using the ModFit software.

Analysis of integrity of the ODC bicistronic transcript.

Ras12V cells were transfected with the bicistronic plasmids and total RNA was isolated and separated on a 1.2% (w/v) agarose-formaldehyde gel, transferred to Hybond N+ membrane (Amersham Biosciences), and UV cross-linked using a Stratalinker (Stratagene). A *Renilla* luciferase cDNA probe was PCR amplified from the bicistronic plasmid on the pcDNA3.0 backbone. The cDNA probe complementary to the 3′ end of *Renilla* luciferase transcript was labeled with [^{32}P]dCTP using the Rad prime DNA radiolabeling kit (Invitrogen). Membranes were exposed to X-ray film.

For reverse transcription-PCR analysis, the empty bicistronic pcDNA3.0 vector or the bicistronic pcDNA3.0 plasmid containing the 303-base ODC 5′UTR was transfected into Ras12V cells. Twenty-four hours after transfection, total RNA was isolated and cDNA was synthesized as described above. A gene-specific reverse primer FFREV (5′-CAGAATGTAGCCATCCATCC-3′) complimentary to the 3′ end of Firefly luciferase coding sequence was used to synthesize the cDNA. Using a forward primer spanning the start codon of *Renilla* luciferase RLATG (5′-GCTAGCCACCATGACTTC-3′) and the FFREV primer, the bicistronic transcript was amplified and analyzed on a 0.8% agarose gel.

For analysis of cryptic promoter activity, the 303-base ODC 5′UTR was cloned into the promoterless Basic pGL3 plasmid (Promega) containing firefly luciferase. The ODC 5′UTR plasmid was compared with the Basic pGL3 vector and to a control pGL3 plasmid (Promega) containing the SV40 promoter. Cells were extracted in lysis buffer (Promega) and luciferase activity was measured using the luciferase assay kit (Promega).

ODC assay and polyamine measurements.

Cells were allowed to adhere for 16 h, inhibitors were added, and cells were extracted in ODC assay buffer 24 and 48 h later. ODC activity was measured by the release of 14-CO_2 from l-[1-^{14}C]ornithine as described previously (**27**). Polyamine content was measured using reverse-phase high-performance liquid chromatography analysis and normalized to total protein (**11**).

Western blots.

Cells were plated and treated with inhibitors, then extracted in a homogenization buffer containing 20 mmol/L HEPES, 2 mmol/L EGTA, 100 mmol/L NaF, 100 mmol/L KCl, 0.2 mmol/L EDTA, 50 mmol/L α-glycerophosphate, 2.5% Triton X-100 and 0.25 g sodium deoxycholate, 1 mmol/L DTT, 1 mmol/L benzamidine, 0.5 mmol/L sodium vanadate, and protease cocktail inhibitor (Calbiochem). Total cellular protein (20 μg) was resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Micron Separations). Membranes were probed using mouse monclonal antibodies against Ras (Upstate), and rabbit polyclonal antibodies against total and phosphorylated AKT (Ser^{473}), total and phosphorylated eIF4E (Ser^{209}), total and phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}), total and phosphorylated 4EBP1 (Ser^{65}), or phosphorylated p70S6K (Thr^{389}; Cell Signaling

Technology). ODC protein was detected using a purified rabbit polyclonal antibody against mouse ODC (**6**) and quantitated using a FluorImager and ImageQuant software (Molecular Dynamics).

Results

Characterization of the transformed morphology of RIE-1 cells.

RIE-1 cells were stably transfected with the constitutively active Ras mutant G12V (Ras12V cells). All the clones expressing Ras12V exhibited characteristics associated with a transformed phenotype. Although RIE-1 cells expressing an empty vector were epithelial, with a flat, round morphology, Ras-expressing clones were fibroblastic and refractile (Supplementary Fig. S1). Ras12V cells were able to form foci on a monolayer, exhibited anchorage-independent growth in soft agar, and lost expression of the epithelial marker E-cadherin (data not shown). Ras clones also show a rapid growth rate, with a doubling-time of 15 to 19 h compared with the doubling time of 28 h in the control cells.

A clone expressing very high levels of Ras (clone 35) and another expressing much lower levels (clone 49) were selected for further analysis. Both the high-Ras and low-Ras clones were found to have increased levels of ODC activity at 24 and 48 h of growth compared with control cells (Supplementary Fig. S1). At 24 h of growth, ODC activity was increased >20-fold in the high-Ras clone and ∼10-fold in the low-Ras clone, suggesting a positive correlation with levels of Ras expressed and activity of ODC. To analyze the effects of such high ODC activity, polyamine content was measured. Compared with the control cells, the high-Ras cells exhibited markedly increased putrescine content (<0.1 nmol/mg protein in control cells compared with 1.75 nmol/mg protein in Ras12V cells), but relatively little change in spermidine and no change in spermine (data not shown). This pattern is similar to that seen in other cell types with high ODC activity and *in vivo* systems with Ras activation (**6**, **28**).

Ras-activated signaling pathways that regulate ODC activity.

Two pathways that have been established as critical to Ras-mediated transformation in RIE-1 cells are those downstream of Raf and PI3K (**29**). To determine the effect of these pathways in regulating ODC, Ras12V cells were treated with LY294002 and rapamycin, which inhibit PI3K and mTOR, respectively, and PD98059, which inhibits MEK. MEK inhibition blocked phosphorylation of ERK1/2 within 90 min without affecting total ERK1/2 (Supplementary Fig. S2). The effect of LY294002 was confirmed by a decrease in the phosphorylated levels of Akt; in the presence of rapamycin, a decrease was observed in the phosphorylated levels of p70S6K and 4EBP1 (Supplementary Fig. S2). When Ras12V cells were grown in the presence of these inhibitors for 24 h (**Fig. 1*A***) or 48 h (data not shown), a 50% to 60% decrease in ODC activity was observed in both the high-Ras clone and low-Ras clone compared with the vehicle-treated controls. Western blot analysis showed that ODC protein was also reduced (**Fig. 1*C***). None of these inhibitors individually was sufficient to reduce ODC to levels comparable with those in parental control cells. Such a drastic inhibition in ODC activity and protein was observed only when both pathways were inhibited (**Fig. 1*B* and *C***). Inhibitor treatment of control cells reduced ODC activity and protein to below the limits of detection.

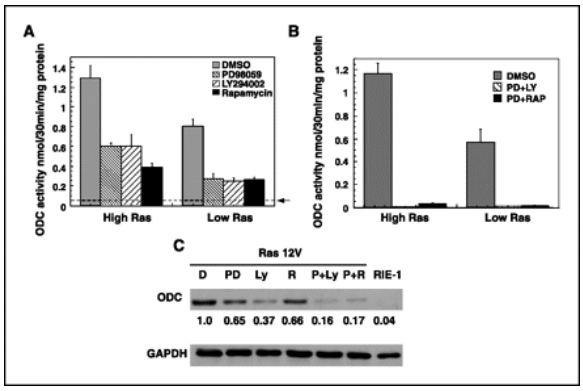

Figure 1. Effect of MEK and PI3K/mTOR inhibition on ODC activity in Ras12V cells. ODC activity for each clone was measured by treating the cells with either the vehicle DMSO, 50 µmol/L PD98059, 20 µmol/L LY294002, or 100 nmol/L rapamycin (*A*), or by using a combination of either 50 µmol/L PD98059 (*PD*) and 20 µmol/L LY294002 (*LY*), or 50 µmol/L PD98059 and 100 nmol/L rapamycin (*RAP*; *B*). *Columns,* average of triplicates per experiment. Experiments were done in duplicate. The ODC activity in control RIE-1 cells (*arrow in A*) was 0.05 nmol/30 min/mg protein. ODC activity in the presence of inhibitors was found to be significantly different from the vehicle controls with a $P < 0.01$ as measured by a one-way ANOVA using Dunnett's multiple correction tests. ODC protein in Ras12V cells (high Ras clone) was also reduced in the presence of inhibitors (*C*). The density of the band corresponding to ODC in DMSO (*D*)–treated Ras12V cells was set at 1.0, and the densities of other bands are expressed as a fraction of that value. The blot was stripped and reprobed with an antibody directed against glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which served as a loading control.

Identification of alternate splice variants present in the mRNA of ODC.

To test if any mutations or alternative splicing could be detected in the ODC sequence in Ras12V cells, total RNA was isolated and reverse transcribed to cDNA. The ODC coding regions, 3'UTR and 5'UTR, were amplified by PCR and sequenced. No alterations induced by Ras transformation in the ODC open reading frame or 3'UTR were observed. Several overlapping sequences were observed in the ODC 5'UTR, which were identified as four splice variants in both Ras12V cells and untransformed controls. These species were 303, 290, 286, and 273 nucleotides in length, and differed from each other in the presence or absence of an additional 13- and 17-base intronic sequence (**Fig. 2*A***). The 17-base intronic sequence is also present in the mouse ODC 5'UTR (data not shown), and a conserved pyrimidine-rich sequence within the 13-base intron is present in the human ODC 5'UTR (**21**).

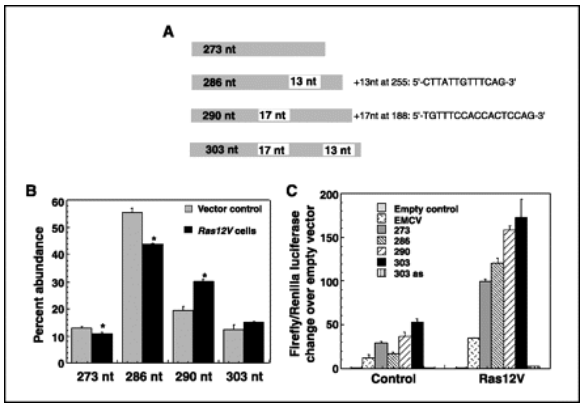

Figure 2. Identification of four splice variants of ODC 5'UTR sequence and measurement of their IRES activities. *A,* lengths of four splice variants of ODC 5'UTR identified in both Ras12V and control RIE-1 cells. Also indicated is the presence or absence of 17- and 13-base intronic sequences. *B,* distribution of splice variants was determined by capillary electrophoresis as described in Materials and Methods. *Columns,* average of two separate experiments with triplicate samples per experiment. Difference in the distribution of the 273-base

variant in Ras12V cells compared with controls was statistically significant (*$P < 0.05$), and also significant for 286- and 290-base variants (*$P < 0.0001$) as calculated by a two-tailed *t* test. *C,* cells were transfected with either the empty Renilla-firefly bicistronic plasmid or with the plasmid containing each of four ODC 5'UTR splice variants inserted between Renilla (cap) and Firefly (cap-independent) luciferase sequences. Luciferase activities were measured as described in Materials and Methods. 303 as, containing the 303-base sequence cloned in antisense orientation, was used as a negative control. An attenuated IRES from the EMCV virus was used as a positive control. *Y axis,* ratios of Firefly to Renilla luciferase activities measured for each plasmid and expressed as a fold change over the ratios measured for the empty bicistronic plasmid. *Columns,* average of two experiments with triplicate samples measured per experiment.

The relative distribution of each variant in both cell lines was analyzed by capillary electrophoresis. Among the four ODC transcripts, the most abundant in both control and Ras12V cells was the 286-base 5'UTR variant (**Fig. 2*B***). However, the distribution trend of these transcripts was different in normal versus transformed cells. When comparing percentage abundance in each cell line, the shorter 5'UTR species of 273 and 286 nucleotides were more abundant in the control cells, whereas the longer 290 and 303 nucleotide species were more abundant in the Ras12V cells. This suggests a Ras-responsive selection of alternative ODC transcripts that contain intronic sequences (**Fig. 2*B***).

Cap-independent translation of ODC.

To assay for the presence of IRES activity in the ODC 5'UTR, the four variant 5'UTR sequences were cloned into a bicistronic plasmid between *Renilla* luciferase and Firefly luciferase, transfected into cells during log-phase growth, and the luciferase activities were measured. The ratios of Firefly (cap-independent) to *Renilla* (cap-dependent) luciferase activities were expressed as fold change over ratios obtained with an empty bicistronic control plasmid. An attenuated viral EMCV sequence reported to exhibit IRES activity (a kind gift from Dr. P. Sarnow) was used as a positive control (**30**). The integrity of the bicistronic transcript was verified by several methods (Supplementary Fig. S3).

All of the ODC splice variants supported IRES-mediated translational activity, and the strongest IRES activity in both normal and transformed cells was exhibited by the 303-base 5'UTR variant (**Fig. 2*C***). Ras transformation had a striking stimulating effect on IRES activity from all of the ODC 5'UTR species, increasing IRES-mediated translation up to 6-fold compared with the control cells (**Fig. 2*C***). A 303-base 5'UTR in the antisense orientation served as a negative length control and as expected exhibited minimal IRES activity (**Fig. 2*C***). Although Ras12V cells exhibited a 5'UTR length-dependent increase in IRES activity, in the control cells the IRES activity of the 286-base 5'UTR variant was consistently significantly lower than the 273-base variant (**Fig. 2*C***).

Effect of inhibitors on IRES-mediated translation of ODC.

The ability of ODC to be translated in a cap-independent manner more efficiently in the Ras-transformed cells puts forth an interesting question as to whether this IRES activity is regulated by Ras signaling. To address this possibility, Ras12V and control cells were transfected with the 303-base bicistronic plasmid, which exhibited the strongest IRES activity, and cells were first treated with PI3K pathway inhibitors. Compared with the vehicle-treated controls, cap-independent translation was decreased by >50% in the presence of LY294002 or rapamycin in the control cells but was decreased only by ~10% to 12% in the Ras12V cells (**Fig. 3*A***).

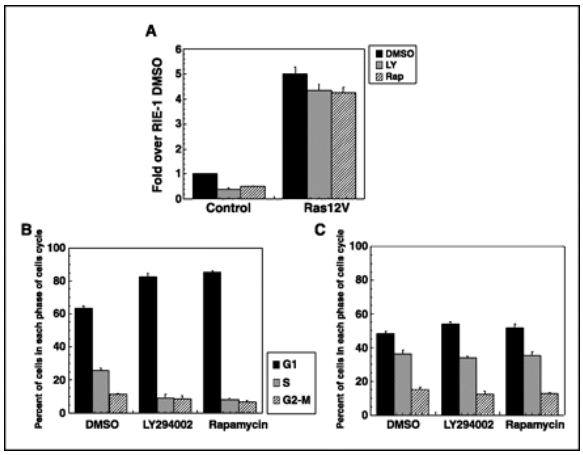

Figure 3. Effect of PI3K/mTOR inhibition on ODC IRES–mediated translational activity and cell cycle progression in Ras12V and parental RIE-1 cells. Cells were transfected and analyzed as described in Materials and Methods. *A,* luciferase assays. *Y axis,* fold change in firefly luciferase (IRES) activity normalized to the activity observed in DMSO-treated parental control cells. *Columns,* average of triplicate samples. The experiments were done in duplicate. Cell cycle progression was analyzed by flow cytometry in RIE-1 cells (*B*) and Ras12V cells (*C*) as described in Materials and Methods. The number of cells in each phase of the cell cycle is represented as a percentage of the total (*Y axis*). *Columns,* average of three independent experiments.

To explain the differential effects of LY294002 and rapamycin in the two cell lines, cell cycle progression was analyzed. The percentages of cells in the G_1, S, and G_2-M phases of the cell cycle were 63%, 26%, and 11%, respectively, in the control cells and 49%, 36%, and 15%, respectively, in the Ras12V cells (**Fig. 3*B* and *C***). In nontransformed cells, LY294002 or rapamycin increased the percentage of cells in the G_1 phase to ~85%, whereas the percentage of cells in the S phase was reduced to 8% to 9%, and in the G_2-M phase to 6% to 8% (**Fig. 3*B***). These inhibitors had no effect on cell cycle distribution in Ras12V cells (**Fig. 3*C***), although Western blot analysis confirmed inhibition of PI3K and mTOR (data not shown). Inhibition of MEK surprisingly caused ODC IRES activity to increase in both the Ras12V and control cells (**Fig. 4*A***). MEK inhibition caused a similar increase in IRES activity when the 273-base ODC 5'UTR was analyzed (data not shown). These results suggest that the stimulatory effect of PD98059 is not dependent on the presence of intronic sequences within the ODC 5'UTR. Contrary to the effects of the inhibitors of the PI3K/mTOR pathway, MEK inhibition did not alter the cell cycle distribution significantly in either cell line (**Fig. 4*B* and *C***).

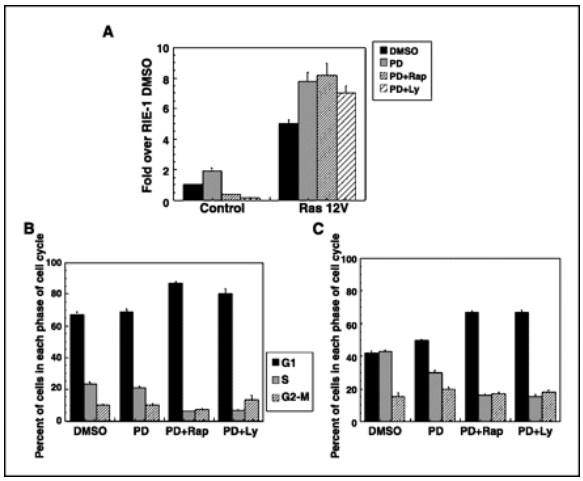

Figure 4. Effects of inhibition of MEK or MEK plus PI3K/mTOR on ODC IRES–mediated translational activity and cell cycle progression in control and Ras12V cells. Control and Ras12V cells were transfected with the empty or 303-base bicistronic plasmid. Immediately after transfection, cells were serum starved for 6 h and serum was

replaced with either DMSO, 50 μmol/L PD98059 (*PD*), PD and 20 μmol/L LY294002 (*LY*), or PD and 100 nmol/L rapamycin (*Rap*). Twenty-four hours after transfection, luciferase activities were measured (*A*) and cell cycle progression was analyzed (*B* and *C*) as described in **Fig. 3**.

Because both the Raf and PI3K pathways cooperate to regulate ODC activity in response to Ras (**11**), ODC IRES activity was measured in the presence of a combination of inhibitors consisting of PD98059 plus LY294002, or PD98059 plus rapamycin. These inhibitor combinations maintained the increased ODC IRES activity in Ras12V cells, but IRES activity was markedly inhibited in control cells compared with their DMSO-treated counterparts (**Fig. 4*A***). Treatment of control cells with the dual inhibitors was accompanied by a marked increase in the number of cells arrested in G_1 compared with DMSO-treated cells (**Fig. 4*B***). On the other hand, all of the inhibitor combinations produced only a minor effect on cell cycle progression in Ras12V cells (**Fig. 4*C***).

To assess the possibility of a compensatory induction in the p38 MAPK pathway in response to MEK inhibition, levels of both phosphorylated and total p38 MAPK, as well as the p38 δ isoform shown to interact with ERK (**31**), were analyzed in the presence of PD98059 or PI3K/mTOR inhibition. No changes in p38 MAPK were observed (Supplementary Fig. S4), making it unlikely that this pathway is affecting ODC IRES activity. Because increased activation of translation initiation factor eIF2-α has been shown to stimulate ODC IRES activity during the G_2-M phase of the cell cycle (**32**), phosphorylated levels of eIF2-α were also analyzed in the presence of all the inhibitors tested. No change was observed in either the total or phosphorylated level of eIF2-α (Supplementary Fig. S4).

Analysis of c-Myc IRES activity.

Among mammalian IRES activities, the one most thoroughly studied is contained within the c-Myc 5'UTR, which has been shown to be responsible for maintaining c-Myc translation under a variety of conditions when cap-dependent protein synthesis is reduced, such as apoptosis, hypoxia, or genotoxic stress (**24**). We examined the activity of the c-Myc IRES in RIE-1 cells and their Ras12V-transformed counterparts. Whereas ODC IRES activity is induced by Ras transformation, we found that c-Myc IRES activity was ∼40% lower in Ras12V cells compared with RIE-1 cells (Supplementary Fig. S4). When Ras12V cells were treated with PD98059, c-Myc IRES activity was not increased significantly (Supplementary Fig. S4). Cap-independent translation of c-Myc is thought to be mediated through the p38 MAPK pathway (**24**). However, we see no activation of p38 MAPK activity, either in response to Ras transformation or MEK inhibition, in this system. This may explain the differences in c-Myc IRES and ODC IRES responses in RIE-1 and Ras12V cells.

Regulation of ODC by eIF4E phosphorylation.

The increase in IRES activity in the presence of MEK inhibition, coupled with the lack of compensation by other pathways, suggest that elements downstream of MEK control ODC IRES activity in Ras12V cells. The Mnk1 and Mnk2 kinases are crucial targets of ERK, which upon activation phosphorylate eIF4E (**19**), and Ras12V cells contain increased levels of phosphorylated eIF4E (data not shown). Therefore, we investigated the role of this phosphorylation in ODC regulation. In the Ras12V cells, phosphorylation of eIF4E was almost completely inhibited in the presence of PD98059, without affecting total eIF4E levels, but neither LY294002 nor rapamycin had a substantial effect on phosphorylation of eIF4E (**Fig. 5*A***). To assess the contribution of eIF4E phosphorylation to ODC IRES activity, the specific Mnk inhibitor CGP57380 (CGP) was used. As expected, Mnk inhibition greatly reduced the phosphorylation of eIF4E without affecting total levels (**Fig. 5*B***). No change in ERK phosphorylation was observed in the presence of Mnk inhibition (**Fig. 5*C***). CGP treatment reduced ODC activity by ∼60% compared with vehicle-treated Ras12V cells (data not shown), suggesting that dephosphorylation of eIF4E is sufficient to inhibit ODC activity and represents a principal target of the MEK pathway in ODC regulation.

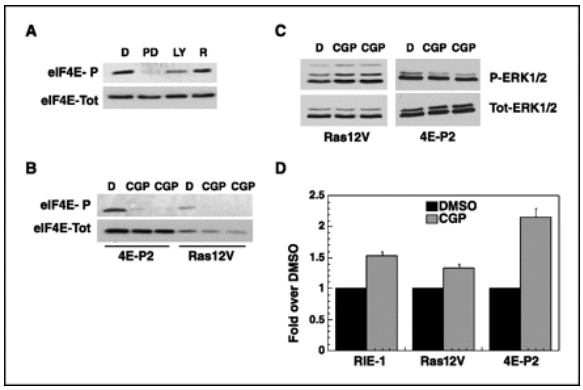

Figure 5. Effect of eIF4E phosphorylation on ODC-IRES–mediated translation in Ras12V cells. Western blot analysis of (*A*) Ras12V cells treated with either DMSO, 50 μmol/L PD98059 (*PD*), 20 μmol/L LY294002 (*LY*), or 100 nmol/L rapamycin (*R*), or (*B* and *C*) both Ras12V and 4E-P2 cells treated with DMSO or 20 μmol/L CGP57380 (*CGP*). Cells were grown in the presence of serum for 24 h and treated with inhibitors in serum-free medium for 2 h. Phosphorylated and total eIF4E (*A* and *B*) or phosphorylated and total levels of ERK (*C*) were analyzed by Western blot in duplicate for each treatment. The experiment was repeated twice to confirm the results. *D,* for luciferase assays, RIE-1, Ras12V, and 4E-P2 cells were transfected with either the empty or 303-base bicistronic plasmid and treated with either DMSO or 20 μmol/L CGP. *Y-axis,* fold change in firefly luciferase (IRES) activity normalized to activity in DMSO-treated cells. *Columns,* average of triplicate samples. The experiment was done in duplicate.

To analyze if the phosphorylation status of eIF4E contributes to ODC IRES regulation, the effect of Mnk inhibition on ODC IRES-mediated translation was analyzed. At a concentration of 20 μmol/L, CGP moderately but significantly increased ODC IRES activity by ~1.5-fold compared with DMSO treatment in both Ras12V cells and control cells (**Fig. 5*D***). These results suggest that dephosphorylation of eIF4E can stimulate ODC IRES activity, while decreasing overall ODC protein synthesis.

To further clarify these results, Mnk inhibition was studied in 4E-P2 cells. These cells represent a good model to analyze the direct effects of eIF4E, as they are transformed by overexpression of eIF4E and consequently circumvent much of the complex regulation associated with Ras overexpression (**13**, **26**). Western blot analysis confirmed that in spite of overexpression of eIF4E, 20 μmol/L CGP was sufficient to significantly block phosphorylation of eIF4E in 4E-P2 cells, without affecting either total eIF4E levels (**Fig. 5*B***) or phosphorylated levels of ERK (**Fig. 5*C***). Treatment of these cells with CGP was also quite effective in inducing ODC IRES–mediated translation, exhibiting more than a 2-fold increase compared with the DMSO-treated controls (**Fig. 5*D***). ODC cap-independent translation exhibited a dose-dependent response to changes in eIF4E phosphorylation obtained by using a range of inhibitor concentrations (**Fig. 6*A***). Cell cycle progression was also analyzed in 4E-P2 cells. Treatment with 20 μmol/L CGP resulted in a 90% reduction in the number of G_2-M phase cells compared with the DMSO-treated controls, yet up-regulated ODC IRES-mediated translation (**Fig. 6*B***).

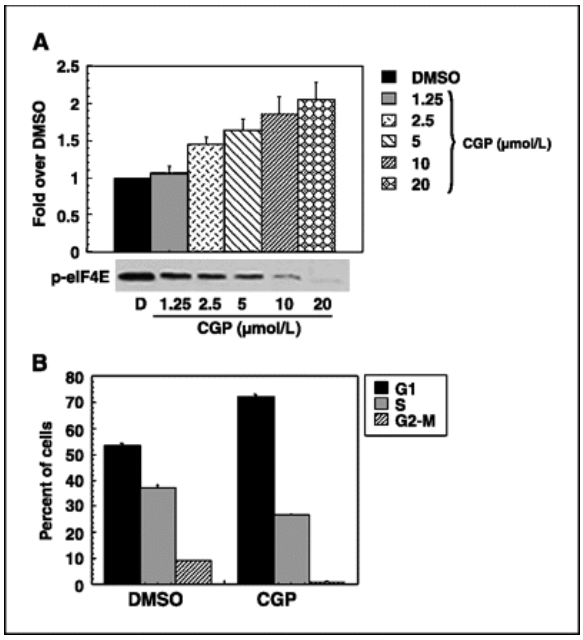

Figure 6. ODC IRES–mediated translation in response to varying doses of the Mnk inhibitor. 4E-P2 cells were transfected with the empty or 303-base bicistronic plasmid. *A,* cells were treated with CGP57380 (*CGP*) at doses ranging from 1.25 to 20 μmol/L, and luciferase activities were measured. *Y-axis,* fold change in firefly luciferase (IRES) activity normalized to the activity observed in DMSO-treated cells. *Columns,* average of triplicate samples and each experiment was done in duplicate. Western blot below displays the effect of varying doses of the Mnk inhibitor on levels of eIF4E phosphorylation. For cell cycle progression analysis (*B*), cells were treated with either DMSO or 20 μmol/L CGP and analyzed as described in **Fig. 3**. *Columns,* average of three independent experiments.

Discussion

Increased ODC activity is necessary for transformation induced either by the direct activation of the *Ras* oncogene or by activation of downstream targets such as MEK (**6**, **14**, **33**, **34**). Although studies in Ras-transformed NIH/3T3 cells have advanced the understanding of ODC regulation in response to Ras, the present study delves into the regulation of ODC in a more pathologically relevant Ras-transformed epithelial cell line. Stable activation of Ras in the RIE-1 cells resulted in about a 20-fold increase in ODC activity, and this increased ODC activity correlated with increased polyamine content, especially in levels of putrescine. Increased polyamine content under typical conditions results in a feedback inhibition of ODC synthesis. However, Ras transformation, as reported previously, results in an absence of such feedback inhibition, thus ensuring the elevated levels of polyamines needed to maintain the transformed state of the cell (**11**). Adding to this complexity of regulation, Ras also seems to regulate ODC activity by cap-independent translational mechanisms in this epithelial cell system. Here, we extend previously reported studies and show that IRES-mediated translation of ODC is both increased by Ras transformation and inversely regulated by components downstream of Raf.

ODC mRNA in rat pancreatic carcinoma cells was reported to contain several 5'UTR splice variants that exhibit IRES activity (**35**). Analysis of the ODC 5'UTR in both RIE-1 and Ras12V cells identified the presence of these four alternate splice variants, which differed from each other in the presence or absence of an additional 13- or 17-base intronic sequence. This is the first report of these splice variants in normal, nontransformed rat cells. Normal pancreatic acinar cells expressed only the intronless 273-base species (**35**), and this was also the species first identified in rat (**36**).

ODC belongs to a unique subset of mRNAs that are characterized by a long GC-rich 5'UTR with a complex secondary structure. Such long structured 5'UTRs are in general inhibitory to cap-dependent translation, but can allow translation under specific conditions through a cap-independent, IRES-mediated mechanism (**21**, **24**, **37**, **38**). Our results confirmed that all of the observed ODC 5'UTR variants contained active IRES sequences, and IRES-mediated activity was markedly activated in cells transformed by Ras. In both the Ras-transformed and nontransformed cells, the presence of both the 17- and 13-base intronic sequences produced the strongest IRES activity. This may be attributed to the presence of a pyrimidine-rich region in both these intronic sequences, with similarities to certain viral IRES elements (**21**).

Intriguingly, several known IRES-transacting factors are also factors associated with aberrant splicing under cancerous conditions (**39**). Our studies found a difference in splice variant abundance, with the 290- and 303-nucleotide species being more abundant in Ras12V cells compared with RIE-1 cells. Although the difference was moderate, it may have a significant functional consequence to overall ODC activity in Ras12V cells, as ODC IRES activity of all the splice variants was clearly increased in response to Ras transformation. Certain IRES *trans*-acting factors such as PTB and HnRNPA1 have been shown to exhibit increased activity in specific types of cancers (**39**). Although neither of these factors was found to regulate ODC IRES activity *in vitro* (**22**), cell-specific analysis must be carried out to identify ODC IRES *trans*-acting factors.

It is noteworthy that the fold increase in ODC IRES activity compared with the empty bicistronic plasmid is more pronounced in both cell lines used in the current studies compared with previous reports studying the rat ODC IRES in HeLa cells (**21**, **22**). A possible explanation is that the HeLa cells are of human origin and these splice variants have been derived from the rat. Earlier studies have specified cell type differences in IRES-regulating factors (**40**). As this study was carried out in RIE-1 cells using splice variants derived from the same cell type, the ODC IRES may be in a more physiologically relevant context. In support of such an observation, the mouse ODC 5'UTR had IRES activity similar to the 273-base species, although it contains the 13-base intronic sequence (data not shown).

Compared with vehicle-treated controls, cap-independent translation was decreased by >50% in the presence of LY294002 or rapamycin in parental RIE-1 cells. This was accompanied by an increase in the number of G_1 phase cells, and a corresponding decrease in S-phase and G_2-M phase cells. These results are in agreement with previous studies suggesting that ODC IRES activity is induced during the G_2-M phase of the cell cycle (**21**), and further suggest that the ODC IRES is positively controlled by the PI3K pathway in nontransformed control cells. In contrast, these inhibitors had no effect on cell cycle distribution in Ras-transformed cells and did not alter IRES activity. The resistance of Ras-transformed cells to cell cycle arrest implies that the PI3K pathway fails to respond appropriately to inhibitory signals, allowing IRES-mediated ODC translation to proceed in the rapidly proliferating Ras-transformed cells. However, inhibition of PI3K or mTOR causes ODC enzyme activity to be reduced by ∼50% to 60% in Ras12V cells, supporting a regulatory role for this pathway in control of cap-dependent ODC translation through 4EBP1 in the Ras-transformed cells.

Inhibition of MEK using PD98059 did not significantly affect cell cycle progression in either cell line, yet surprisingly increased ODC IRES activity by ∼2-fold in both control and Ras12V cells. These results suggest that the ODC IRES can be negatively regulated by the Raf/MEK/ERK pathway, and this effect seems to be independent of the transformed status of the cells. It is possible that a specific *trans*-acting factor is repressed by this pathway and is released upon inhibition. A recent study showed enhancement of hepatitis C viral IRES-mediated translation in the presence of PD98059 (**41**). To expand these results, cells were treated simultaneously with the MEK inhibitor and either LY294402 or rapamycin. IRES activity in control cells was inhibited by this treatment, suggesting that the PI3K pathway may have a dominant role in regulating the ODC IRES in the nontransformed cells. On the other hand, the increased ODC IRES activity was maintained in the

Ras12V cells subjected to either inhibitor combination, suggesting the Raf-controlled pathway dominates in Ras-transformed cells.

It has been shown that both Myc and cyclin D1 IRES elements are positively regulated by p38 MAPK– and ERK-dependent pathways, but only under conditions of quiescent Akt activity, which induces these pathways (**42**). We found p38 MAPK levels to be very low in both RIE-1 and Ras12V cells, and Akt activity is induced in Ras12V cells. Indeed, we saw a reduction in c-Myc IRES-mediated luciferase translation in Ras12V cells compared with RIE-1 cells, and no change when Ras12V cells were treated with PD98059. Although there have been no reports regarding the effect of Ras transformation on cap-independent c-Myc translation, the c-Myc IRES is most active during apoptosis (**24**), and MEK inhibition does not induce apoptosis in these cells.

The Mnk1/2 kinases are activated by ERK and/or p38 MAPK, with Mnk1 regulating inducible phosphorylation of eIF4E and Mnk2 affecting basal phosphorylation levels (**43**). The low p38 MAPK levels in both cell lines described here suggest that Mnk1 seems to be controlled almost entirely by the Raf/MEK/ERK pathway. Thus, using an Mnk inhibitor would replicate the effects of PD98059 on phosphorylation of eIF4E, without affecting the upstream components of the Raf/MEK/ERK pathway. Interestingly, inhibition of Mnk enhanced ODC IRES activity significantly, suggesting that changes in eIF4E phosphorylation contribute to ODC IRES regulation.

To further understand the role of eIF4E phosphorylation in regulating ODC IRES activity, 4E-P2 cells were treated with the Mnk inhibitor. These cells stably overexpress eIF4E, resulting in cellular transformation, and we and others have shown that ODC translation is increased as a result of this overexpression (**13**, **26**). When Mnk was inhibited in these cells, we observed a dose-dependent increase in ODC IRES activity that was directly correlated with levels of phosphorylated eIF4E. Such a robust effect of eIF4E dephosphorylation on IRES-mediated translation of ODC in these cells points to a direct relationship between eIF4E phosphorylation and regulation of the ODC IRES, which can only be inferred in the Ras-transformed cells, due to the multitude of downstream targets activated by Ras.

The role of phosphorylation in regulating the functions of eIF4E is still the topic of debate, especially because Mnk1/2 double-knockout mice did not reveal any significant growth or developmental defects (**43**). However, these findings do not negate the possibility of functional involvement of phosphorylation of eIF4E in specific physiologic situations (**43**). Dephosphorylation of eIF4E was correlated previously with stimulating the IRES activity of an egg-laying hormone in *Aplysia* neurons after an afterdischarge (**44**, **45**). Phosphorylation of eIF4E is generally thought to decrease its affinity for the cap; however, this has been interpreted to have positive and negative effects on overall protein translation (**46**– **49**). Inhibition of cap-dependent translation upon eIF4E dephosphorylation may increase the availability of specific translation initiation factors such as eIF4G and eIF4A to carry out cap-independent translation.

In summary, the studies described here show that cap-independent translation of ODC can be activated in response to Ras transformation and is differentially controlled by elements downstream of Ras. The IRES element responds to the phosphorylation status of eIF4E, and dephosphorylation of eIF4E either by MEK or Mnk1/2 inhibition increases ODC IRES activity. In situations where both the Raf/MEK/ERK and PI3K/mTOR pathways are inhibited in normal cells, ODC IRES activity is very low and most cells arrest in G_1. However, when both of these pathways are inhibited in Ras-transformed cells, cell cycle arrest does not occur and ODC IRES activity is induced, thus helping to maintain high ODC activity. Although these studies do not address the relative contribution of cap-dependent versus cap-independent translation of ODC in Ras-transformed cells, even a minor contribution to ODC translation by a cap-independent mechanism under transformed conditions may help ensure maintenance of elevated polyamine content in these rapidly growing cells.

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