Interaction of asparagine and EGF in the regulation of ornithine decarboxylase in IEC-6 cells

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Ray, Ramesh M., Mary J. Anne Viar, Tarun B. Patel, and Leonard R. Johnson. Interaction of asparagine and EGF in the regulation of ornithine decarboxylase in IEC-6 cells. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G773–G780, 1999.—Our laboratory has shown that asparagine (ASN) stimulates both ornithine decarboxylase (ODC) activity and gene expression in an intestinal epithelial cell line (IEC-6). The effect of ASN is specific, and other A- and N-system amino acids are almost as effective as ASN when added alone. In the present study, epidermal growth factor (EGF) was unable to increase ODC activity in cells maintained in a salt-glucose solution (Earle’s balanced salt solution). However, the addition of ASN (10 mM) in the presence of EGF (30 ng/ml) increased the activity of ODC 0.5- to 4-fold over that stimulated by ASN alone. EGF also showed induction of ODC with glutamine and α-aminobutyric acid, but ODC induction was maximum with ASN and EGF. Thus the mechanism of the interaction between ASN and EGF is important for understanding the regulation of ODC under physiological conditions. Therefore, we examined the expression of the ODC gene and those for several protooncogenes under the same conditions. Increased expression of the genes for c-jun and c-Fos but not for ODC occurred with EGF alone. The addition of ASN did not further increase the expression of the protooncogenes, but the combination of EGF and ASN further increased the expression of ODC over that of ASN alone. Western analysis showed no significant difference in the level of ODC protein in Earle’s balanced salt solution, ASN, EGF, or EGF plus ASN. Addition of cycloheximide during ASN and ASN plus EGF treatment completely inhibited ODC activity without affecting the level of ODC protein. These results indicated that 1) the increased expression of protooncogenes in response to EGF is independent of increases in ODC activity and 2) potentiation between EGF and ASN on ODC activity may not be due to increased gene transcription but to posttranslational regulation and the requirement of ongoing protein synthesis involving a specific factor dependent on ASN.

epidermal growth factor; polyamines; amino acids; c-Fos; c-jun; enzyme activity

The polyamines spermidine and spermine and their precursor putrescine are required for cell growth and proliferation of eukaryotic cells (7, 40, 47). The intracellular levels of polyamines are highly regulated and primarily depend on the activity of ornithine decarboxylase (ODC; EC 4.1.17), which catalyzes the first rate-limiting step in polyamine synthesis (25–27). ODC activity is regulated differently in different tissues and cells, but, in general, activity increases dramatically and transiently in response to trophic agents, other proliferative stimuli, and damage (7, 11, 13, 18, 27). For these reasons, there is considerable interest in the regulation of the activity of ODC, and the enzyme has been investigated at the level of gene transcription, mRNA translation, protein turnover, and inhibitors of enzyme activity (2, 12, 14, 18, 36).

For a number of years, our laboratory has been interested in the role of polyamines in the growth and repair of the gastrointestinal mucosa and has examined these processes in both rats and cultured, normal intestinal epithelial cells. The mammalian gastrointestinal epithelium is especially suited to study the control of proliferation because of its rapid and continuous renewal. Inhibition of ODC by α-difluoromethylornithine depletes polyamines and inhibits the growth of cultured IEC-6 cells, a nontransformed line originally developed from fetal rat crypt cells (29), and prevents the normal repair of mucosal stress ulcers (45, 46, 49). The addition of exogenous polyamines in both model systems restores cell growth and healing to normal levels.

Basal levels of ODC are quite low, but enzyme activity increases rapidly and dramatically in response to the appropriate stimuli. The enzyme has one of the shortest half-lives of any mammalian enzyme (2, 3). Stimulation of ODC activity in small intestinal mucosa by luminal contents has been well documented. Among normal dietary constitutents, some amino acids have been shown to markedly increase ODC activity and mucosal growth when administered intragastrically (22, 24). In 1971, Fausto (7) showed that some amino acids could increase ODC activity in regenerating liver. Chen and Canellakis (3) and Chen and Chen (4) examined the ability of amino acids to induce ODC activity in cells incubated in a simple salt-glucose solution, thus eliminating stimulation from growth factors present in serum or other amino acids and nutrients normally found in incubation medium. They showed that ODC activity was increased in neuroblastoma cells by asparagine (ASN) and glutamine. Additional studies by the same group (32, 33, 42) showed that, although ASN was the most effective, other amino acids transported by one of the Na+-dependent systems could also induce ODC activity. Induction of ODC activity was shown to be dependent on Na+ and independent of metabolism or the ability of the amino acid to be incorporated into protein. Of particular interest was the finding, in a variety of cell types, that the induction of ODC by hormones and growth factors required the presence of ASN or a similar amino acid (32, 33). Neither epidermal growth factor (EGF), nerve growth...
factor, platelet-derived growth factor, nor insulin was able to induce ODC activity in a salt-glucose solution unless ASN was present in the medium. Wang et al. (48) have shown previously that ASN increases ODC activity and DNA synthesis in IEC-6 cells in growth medium. The increase was accompanied by an increase in ODC mRNA, which was due partly to a decrease in the rate of degradation. EGF also stimulated ODC activity in these cells as well as increased growth and levels of several protooncogenes. Expression of some protooncogenes in IEC-6 cells is inhibited by polyamine depletion (47).

The primary purpose of these studies was to examine the interaction between EGF and ASN in the regulation of ODC activity in IEC-6 cells. We demonstrate that EGF does not increase ODC activity when given in a salt-glucose solution, whereas ASN is able to do so. Furthermore, EGF does potentiate the effects of ASN. We also examined this process of potentiation to discern at which level it occurs. As the studies progressed, it became obvious that we could use the salt-glucose model as a tool to examine whether the effects of EGF require the induction of ODC activity. Thus we show that EGF increases the expression of c-fos and c-jun mRNA in a salt-glucose solution, indicating that this stimulation does not require increases in ODC activity. Two different mechanisms of activation of ODC are indicated because ASN and EGF show potentiation, and this was proven with rapamycin, which blocks serum- and EGF-induced enzyme activity but does not alter the effect of ASN.

MATERIALS AND METHODS

Chemicals and supplies. Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture media and dialyzed fetal bovine serum (FBS) were from GIBCO BRL (Grand Island, NY). Biochemicals were purchased from Sigma (St. Louis, MO). L-[1-14C]ornithine (specific activity 51.6 mCi/mmol) was purchased from DuPont NEN (Boston, MA). The primary antibody was an affinity-purified rabbit polyclonal antibody against ODC and was a kind gift from Dr. M. K. Haddox (University of Texas at Houston) (9). Mouse monoclonal antibody against ODC was purchased from Sigma. Western blot detection systems were purchased from DuPont NEN.

Cell culture. The IEC-6 cell line was obtained from the American Type Culture Collection (Manassas, VA) at passage 13. The stock was maintained in 1 T-150 flasks in a humidified, 37°C incubator in an atmosphere of 90% air-10% CO2. The medium consisted of Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL) with 5% heat-inactivated FBS, 10 µg insulin, and 50 µg gentamicin sulfate/ml. The stock was passaged weekly at 1:20 and fed three times per week. Passages 15–20 were used in the experiments.

Experimental protocol. For most experiments, the cells were taken up with 0.05% trypsin plus 0.53 mM EDTA-4Na in Hanks' balanced salt solution without calcium and magnesium. They were counted in a hemocytometer and plated (day 0) at 6.25 x 10⁶ cells/cm² in DMEM plus 5% dialyzed FBS, 10 µg insulin, and 50 µg gentamicin sulfate/ml. The cells were fed on day 2. On day 3, the medium was removed, the cells were washed once with Hanks' balanced salt solution, and the medium was replaced with serum-free DMEM with 10 µg insulin and 50 µg gentamicin sulfate/ml. On day 4, the medium was removed, the cells were washed once with Hanks' balanced salt solution, and the medium was replaced with Earle's balanced salt solution (EBSS) containing whatever additions were required by the experiment. For experiments involving cell harvesting for Northern blot analysis, the protocol was modified slightly as follows. The serum starvation on day 3 was done in EBSS rather than in DMEM, and, on day 4, treatments were added as concentrated solutions directly into EBSS without changing the medium.

ODC assay. The activity of the enzyme ODC was assayed with a radiometric technique in which the amount of [14C]CO₂ liberated from DL-[1-14C]ornithine was estimated (31). Briefly, after experimental treatment, the dishes were placed on ice; the monolayers were washed three times with cold Dulbecco's PBS (DPBS); and 0.5 ml of 1 mM Tris buffer (pH 7.4) containing 1 mM EDTA, 0.05 mM pyridoxal phosphate, and 5 mM dithiothreitol was added. The cells were frozen at −80°C until assayed. At the time of assay, the cells were thawed on ice, scraped into Microfuge tubes, sonicated, and centrifuged at 12,000 g at 4°C for 10 min. The ODC activity of an aliquot of supernatant was incubated in a stopped tube in the presence of 6.8 mmoles of [14C]ornithine for 15 min at 37°C. The [14C]CO₂ liberated by the decarboxylation of ornithine was trapped on a piece of filter paper impregnated with 20 µl of 2 N NaOH, which was suspended in a center well above the reaction mixture. The reaction was stopped by the addition of TCA to a final concentration of 5%. The [14C]CO₂ trapped in the filter paper was measured by liquid scintillation spectrometry. Aliquots of the supernatant were assayed for total protein with the method described by Bradford (1). Enzymatic activity is expressed as picomoles of CO₂ per hour per milligram of protein.

RNA isolation and Northern blot analysis. Total RNA was extracted with a guanidium isothiocyanate solution and purified by CsCl density gradient ultracentrifugation as described by Chirgwin et al. (6). Briefly, the monolayer of cells was washed with DPBS and lysed in 4 M guanidium isothiocyanate. The lysates were centrifuged through a 5.7 M CsCl cushion at 150,000 g at 18°C for 20 h. After centrifugation, the supernatant was aspirated, and the resulting RNA pellet was dissolved in 10 mM Tris-HCl (pH 7.4) containing 5 mM EDTA and 1% sodium dodecyl sulfate (SDS). The purified RNA was precipitated from the aqueous phase by the addition of 0.1 vol of 3 M sodium acetate and 2.5 vol of ethanol in sequence. Final RNA was dissolved in water and estimated from its ultraviolet absorbance at 260 nm, with a conversion factor of 40 optical density units. Total RNA (30 µg) was denatured and fractionated electrophoretically with a 1.2% agarose gel containing 3% formaldehyde and transferred by blotting to a nitrocellulose membrane. The blot was prehybridized for 24 h at 42°C with 5× Denhardt's solution, 5× saline-sodium citrate (SSC), 50% formamide, 25 mM potassium phosphate, and 50 µg/ml of denatured salmon sperm DNA. Hybridization was carried out overnight at 4°C in the same solution containing 10% dextran sulfate and DNA probes, which were labeled with [α-32P]dCTP with a standard nick translation procedure. Blots were washed once with 1× SSC-0.1% SDS at room temperature for 10 min, followed by two changes of 0.25× SSC-0.1% SDS, the first at 42°C for 25 min and the second at room temperature for 10 min. After the final wash, the membrane was autoradiographed with intensifying screens at −80°C.

Western blot analysis of ODC protein. After experimental treatments, IEC-6 cells were washed twice with DPBS. The DPBS was removed, 500 µl of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1
mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, and 1 µg/ml each of aprotinin, leupeptin, and pepstatin) were added, and the plates were rotated at 4°C for 30 min and scraped. The cell lysate was centrifuged at 10,000 rpm for 10 min, and the clear supernatant was used to determine the protein concentration by the method of Bradford (1). Total cell protein (500 µg) was immunoprecipitated with anti-mouse ODC antibody separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane for Western blotting with anti-rabbit ODC antibody. Immune complexes were visualized with a chemiluminescence detection system (DuPont NEN).

[3H]leucine incorporation. Cells were grown in DMEM as described in Experimental protocol. The cells were then incubated in EBSS with ASN and/or EGF, 5 µCi/ml of [3H]leucine were added after 2.5 h, and the cells were further incubated for 30 min. The cells were washed twice with DPBS and lysed with radioimmunoprecipitation assay buffer described in Western blot analysis of ODC protein. The amount of radioactivity in the cell extract was determined. Proteins equivalent to 100,000 counts/min from each sample were precipitated by 10% TCA and separated by 10% SDS-PAGE. Detection of radioactive proteins was carried out with the use of ENHANCE (DuPont NEN) reagent following the manufacturer’s instructions.

RESULTS

Synergistic effect of ASN and EGF on ODC activity. IEC-6 cells were cultured in DMEM as described in MATERIALS AND METHODS. The medium was replaced with EBSS containing varying concentrations of ASN with and without 30 ng/ml of EGF. Basal ODC activity was nearly undetectable. ASN in doses ranging from 1 to 100 mM significantly induced ODC activity, with a peak effect occurring at a concentration of 10 mM (Fig. 1). EGF by itself had no effect on enzyme activity but when given with ASN caused a significant increase in ODC activity over and above that induced by the comparable dose of ASN. As shown in Fig. 2, glutamine (N transport system) and α-aminoisobutyric acid (AIB; A type) also increased ODC activity, which was increased further by EGF. The potentiation by EGF, however, was greater with ASN than with these other two amino acids. Amino acids transported by other systems were ineffective because valine (L type) and lysine (Y type) caused no increase in ODC activity.

Fig. 2. Effect of ASN and other amino acids on induction of ODC activity. ASN, glutamine (GLN), α-aminoisobutyric acid (AIB), valine (VAL), and lysine (LYS) were added to EBSS at a concentration of 10 mM in presence and absence of EGF (30 ng/ml), and ODC activity was determined after a 3-h incubation. Values are means ± SE; n = 6 cultures. *P < 0.05 compared with corresponding control value.

Effects on ODC mRNA and protein levels. To gain some insight into the synergy between ASN and EGF in their regulation of ODC activity, we examined ODC mRNA and protein levels under the same set of experimental conditions. Figure 3 shows that ASN increased ODC mRNA more than threefold, whereas EGF had little effect by itself. When EGF was added to EBSS containing ASN, however, mRNA levels increased more than fivefold. Even though EGF had no effect on ODC activity or ODC mRNA levels, it increased c-fos and c-jun mRNA. ASN had no effect on the mRNA levels of these two protooncogenes, and there was no further increase in mRNA when ASN and EGF were combined.

Western analysis of ODC protein showed little correlation with the observed changes in either enzyme activity or ODC mRNA (Fig. 4). IEC-6 cell extracts contained a protein band corresponding to the 53-kDa molecular mass of a monomer of ODC (Fig. 4A). The identity of this band was confirmed by loading preimmune serum onto the gel, which showed no band corresponding to ODC, and by immunoprecipitation with anti-mouse ODC antibody (Fig. 4B). Results in Fig. 4 show no difference in ODC protein in any of the treatment groups compared with the control group or with each other. These data were further supported by the incorporation of [3H]leucine into the cytoplasmic fraction of IEC-6 cells under the same experimental conditions. Although EGF increased the overall rate of protein synthesis, none of the treatment groups showed an apparent change in the intensity of any particular band and specifically in the 53-kDa region (Fig. 5). Immunoprecipitation of ODC from the labeled extracts failed to detect ODC protein on the same gel.

Stability of ODC activity, mRNA, and protein. As a possible explanation for the combined effects of ASN and EGF, we next examined whether EGF altered the stability of ODC activity, mRNA, and protein in the presence of ASN. As shown in Fig. 6, the addition of EGF to EBSS containing ASN increased the half-life of ODC activity from 46 to 66 min. Because ODC activity

Fig. 1. Dose-dependent induction of ornithine decarboxylase (ODC) activity in IEC-6 cells exposed to asparagine (ASN) in presence (+) and absence (−) of epidermal growth factor (EGF; 30 ng/ml). Cells were grown in Dulbecco’s minimal essential medium as described in Experimental protocol. Cells were then incubated in Earle’s balanced salt solution (EBSS) with ASN and/or EGF, and ODC activity was determined after 3 h of incubation. Values are means ± SE; n = 3 cultures. *P < 0.05 compared with corresponding control value.
is undetectable in EBSS alone or in EBSS to which EGF has been added, we were only able to compare these two groups. Examination of the half-life of ODC mRNA under the same conditions showed no difference between ASN alone and ASN plus EGF (Fig. 7).

Although treatment with cycloheximide for 2 h resulted in 84 and 76% losses in ODC activity in cells stimulated with ASN and ASN plus EGF, respectively, Western blot analysis showed no difference in the levels of ODC protein in the same experiment (Fig. 8). In the absence of cycloheximide, cells stimulated by ASN alone lost 45% of their ODC activity in 2 h, whereas those stimulated with both ASN and EGF lost only 20% of their activity. Figure 8 also shows the disappearance of the ODC protein band from preimmune serum.

Effect of cycloheximide and actinomycin D on the induction of ODC activity. Even though changes in mRNA levels correlated fairly well with the induction of ODC activity after ASN, EGF, and the combination of the two, it is obvious from Fig. 4 that these were not reflected by the amount of ODC protein present. In all cases, including unstimulated control cells, the levels of enzyme protein were approximately the same. These data suggest the involvement of posttranslational processes in the induction of ODC activity. To determine the involvement of protein synthesis in the induction of enzyme activity, cells were exposed to cycloheximide at the same time that they were stimulated by either ASN, that EGF, or the two agents combined. Table 1 shows that cycloheximide prevented any enzyme stimulation by ASN and by the combination of ASN and EGF.

Fig. 3. Effect of ASN and/or EGF on expression of ODC and protooncogene (c-fos and c-jun) mRNA in IEC-6 cells. Cells were stimulated by ASN (10 mM) in presence and absence of EGF (30 ng/ml). A: RNA was harvested after a 3-h incubation in EBSS (Control), ASN, EGF, or ASN + EGF and analyzed by Northern blotting with cDNA probes. B: Quantitative analysis of Northern blots was carried out by densitometry and relative levels of mRNA for ODC were corrected for RNA loading by densitometry of α-actin. Results of a representative experiment are shown.

Fig. 4. Western analysis of ODC protein level in IEC-6 cells treated as described in Fig. 3. Whole cell extracts (100 µg of protein; A) and immunoprecipitated ODC protein from the above cell extracts (B) were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane for immunodetection with anti-rabbit polyclonal ODC antibody as described in MATERIALS AND METHODS. Results of a representative experiment are shown; n = 3 cultures. Nos. on left, molecular mass markers.

Fig. 5. A: [3H]leucine incorporation in IEC-6 cells in EBSS (Control) alone and with ASN, EGF, or ASN + EGF. cpm, Counts/min. B: Fluorogram of [3H]leucine-labeled extract of IEC-6 cells in EBSS alone and with ASN, EGF, or ASN + EGF. Nos. on left, molecular mass of marker proteins. Arrow, position corresponding to ODC monomer as revealed from immunoprecipitated samples of the above extracts loaded on the same gel.
To distinguish the mechanisms of the induction of ODC activity, investigators added growth factors or serum to cells in culture medium that, of course, contained a full complement of amino acids. As a result, all mechanisms were activated simultaneously.

In our present experiments, ASN caused a dose-dependent increase in ODC activity and EGF was without effect when given by itself. However, EGF significantly increased the stimulation provided by ASN (Fig. 1). Although other amino acids transported by A- and N-type systems increased ODC activity and showed synergy with EGF, ASN was the most effective (Fig. 2). All of these results (Figs. 1 and 2) agree with previous reports in other cell types (4, 5, 15, 19).

A nonmetabolizable amino acid analog, AIB, also stimulated ODC activity, indicating that the effects of amino acids are direct and not caused by subsequent processing. The response to AIB was also increased further by the addition of EGF (Fig. 2). These data indicate that the synergism with EGF is specific for A- and/or N-type amino acids and suggests that ASN or a similar amino acid must be present for the maximal induction of ODC activity. Kandil et al. (17) have also shown that glutamine and ASN stimulated ODC activity and proliferation but that AIB was ineffective in a porcine jejunal enterocyte cell line. Chen and Chen (5) found a greater induction of ODC activity in ras-transformed 3T3 cells compared with their nontransformed counterparts, suggesting the involvement of an additional cellular factor for maximal induction.

The effects of ASN and EGF on the level of ODC mRNA in EBSS were qualitatively similar to their effects on ODC activity. ASN by itself increased ODC mRNA 3.5-fold, whereas EGF stimulated a nonsignificant 40% increase. In the presence of ASN, however, EGF further increased ODC mRNA to 5.5 times the basal level (Fig. 3). Upregulation of primary growth response genes, the protooncogenes c-fos and c-jun, which encode transcription factors, in response to EGF may result in activation of the ODC gene via activator protein-1 (23). Increased levels of ODC gene transcription and enzyme activity by c-myc have been demonstrated in BALB/c, 3T3, and rat la fibroblasts (43). In our study, however, EGF increased c-fos and c-jun mRNA expression even though it had no significant effect on ODC mRNA. Thus the stimulation of these protooncogenes by EGF does not depend on its ability to increase ODC activity. In contrast, although ASN signifi-

Thus the synthesis of new protein is required for the induction of ODC activity. Table 1 also shows that enzyme activation is prevented by actinomycin D, indicating that transcription is also required for the stimulation of ODC activity.

Effect of rapamycin on the induction of ODC activity. To distinguish the mechanisms of the induction of ODC by serum and ASN in a salt-glucose solution, basal ODC activity was induced by 10 mM ASN and 10% FBS in the presence and absence of 100 nM rapamycin, a selective repressor of translation initiation of an mRNA with a polypyrimidine-rich tract that includes ODC mRNA. Figure 9 shows that 10 mM ASN and 10% FBS increased ODC activity >800- and 200-fold, respectively, when added alone for 3 h. The addition of rapamycin along with 10% FBS significantly repressed the ODC induction by 80%; however, ASN-mediated induction was not affected by rapamycin. This result clearly indicates that the mechanism of ODC induction in salt-glucose solution is a distinct phenomenon and further supports the posttranslational control of ODC enzyme activity induction by ASN.

DISCUSSION

A variety of evidence indicates that at least two different mechanisms are involved in the stimulation of ODC activity. Several stimuli increase ODC mRNA in both tissues and cultured cells, but, in many cases, the increase in mRNA does not account for the magnitude of induction of ODC activity (44). Ginty et al. (8) found that increased levels of active ODC protein and enzyme activity were sensitive to putrescine and to a calmodulin antagonist, whereas other processes regulating ODC mRNA were insensitive to these compounds. The findings that ASN but not growth factors can increase ODC activity in a salt-glucose solution and that growth factors will, nevertheless, potentiate the induction of enzyme activity by ASN also argue strongly for independent signaling pathways for the regulation of ODC.

We conducted all of the current experiments in EBSS with glucose added to separate the effects of ASN from those of growth factors. In previous experiments designed to investigate the mechanism of regulation of ODC activity, investigators added growth factors or serum to cells in culture medium that, of course, contained a full complement of amino acids. As a result, all mechanisms were activated simultaneously.

In our present experiments, ASN caused a dose-dependent increase in ODC activity and EGF was without effect when given by itself. However, EGF significantly increased the stimulation provided by ASN (Fig. 1). Although other amino acids transported by A- and N-type systems increased ODC activity and showed synergy with EGF, ASN was the most effective (Fig. 2). All of these results (Figs. 1 and 2) agree with previous reports in other cell types (4, 5, 15, 19).

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cantly increased both ODC mRNA and activity, it had no effect on the expression of c-fos and c-jun. Taken together, our Northern analyses indicate a mutual independence between ODC induction and protooncogene transcription in IEC-6 cells.

The significant potentiation of ASN-induced ODC activity by EGF could be due to a variety of events, including posttranscriptional stabilization of ODC mRNA, recruitment of ODC mRNA from a nontranslatable pool, ODC enzyme synthesis (translation), or posttranslational modification (activation) of the enzyme. Regulation of ODC is cell or tissue specific and may involve one or more of the above mechanisms, depending on the polyamine requirements for function of the cells or tissue in question (16, 20). The stability of ODC mRNA in cells treated with both ASN and EGF appeared to be the same as in those treated with ASN alone (Fig. 7), ruling out posttranscriptional stabilization of ODC mRNA as an explanation for the synergistic induction of ODC activity.

Changes in translation have been suggested in several other systems for the regulation of ODC activity (39). ODC exists in an equilibrium between active dimers and inactive monomers (38, 41). In its active form, mammalian ODC is a homodimer, whereas the 53-kDa monomer retains no enzymatic activity. In a recent study, Pegg and Williams-Ashmann (28) presented biochemical evidence that the active site of mammalian ODC interfaces between the two subunits that form the active dimer. Moreover, Tobias and Kahana (41) showed that each subunit has a split active site, with the two residues participating in the active site being located on different segments. These observations established the relationship between structural properties and enzymatic activity and suggest that the level of the 53-kDa monomer does not indicate the level of active ODC protein in the cell extract but only the level of inactive ODC protein. The level of ODC protein measured as the 53-kDa protein did not change significantly in cells treated with ASN or in those treated with ASN plus EGF compared with EBSS or EGF alone (Fig. 4). This observation was further supported by [3H]leucine incorporation, which showed that stimulation of ODC activity in IEC-6 cells by ASN or ASN plus EGF was not due to the stimulation of general protein synthesis. EGF significantly increased [3H]leucine incorporation 52% compared with that in the control group (Fig. 5A). Immunoprecipitation of ODC from [3H]leucine-labeled cell extracts failed to show an ODC protein band corresponding to 53 kDa. A fluorogram of labeled protein showed no gross changes in the intensity of labeling of any protein band in the 53-kDa region (Fig.

Table 1. Effect of cycloheximide and actinomycin D on ODC activity

<table>
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<tr>
<th>Treatment</th>
<th>Cycloheximide (50 µg/ml)</th>
<th>Actinomycin D (5 µg/ml)</th>
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<tbody>
<tr>
<td>Without</td>
<td>With</td>
<td>Without</td>
</tr>
<tr>
<td>Control</td>
<td>3.14 ± 1.2</td>
<td>ND</td>
</tr>
<tr>
<td>ASN</td>
<td>445.5 ± 79.1</td>
<td>ND</td>
</tr>
<tr>
<td>EGF</td>
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<td>ND</td>
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<tr>
<td>ASN + EGF</td>
<td>1,705.2 ± 81.6</td>
<td>2.86 ± 5.2</td>
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Values are means ± SD; n = 3 cultures. ODC, ornithine decarboxylase; ASN, asparagine; EGF, epidermal growth factor; ND, not detected. Cycloheximide and actinomycin D were added with treatments. ODC activity was determined after 3 h.

Fig. 8. Stability of ODC protein in IEC-6 cells stimulated with ASN or ASN + EGF. Extracts (500 µg of protein) from cells stimulated with ASN (10 mM) or ASN + EGF (30 ng/ml) for 3 h (0 h) with and without cycloheximide (50 µg/ml) treatment (2 h) were immunoprecipitated and subjected to Western blotting analysis as described in Fig. 4.

Fig. 9. Effect of rapamycin on induction of ODC activity by 10% fetal bovine serum (FBS) or ASN. Cells were grown in DMEM as described in Experimental protocol. Cells were then incubated in EBSS with ASN or 10% FBS in presence and absence of 100 nM rapamycin (Rapa), and ODC activity was determined after 3 h of incubation. Values are means ± SE; n = 6 cultures. *P < 0.05 compared with corresponding control value.
5B). These results clearly indicate that the induction of ODC activity by ASN or ASN plus EGF does not involve new ODC protein synthesis.

Poor translatability of ODC mRNA in vitro has also been reported and suggested to be due to an extensive secondary structure at the 5'-end of the ODC mRNA. The eukaryotic initiation factor eIF-4F participates in the melting of mRNA secondary structures (10, 21). Overexpression of eIF-4F increases the translation efficiency of mRNA with a secondary structure, including ODC mRNA (37). It is possible that the induction of ODC by ASN plus EGF may involve eIF-4F and increased rates of translation of the ODC gene. Seidel and Ginty (34) have shown that the addition of FBS to the medium induced ODC activity in IEC-6 cells and that this was accompanied by a parallel increase in the level of ODC protein as determined by [α-3H]difluoromethylornithine binding, which measures only active enzyme protein. The addition of putrescine along with serum decreased ODC activity, with a concomitant decrease in ODC protein content. Seidel and Ragan (35) further established the role of eIF-4F in the induction of ODC activity in IEC-6 cells by using rapamycin, a specific inhibitor of eIF-4F-mediated translation repression. Surprisingly, only 50% of the serum-induced ODC activity was inhibited by rapamycin, strongly suggesting that more than one mechanism is involved in the induction of ODC activity and that the mechanism depends on the type of stimulus. This observation prompted us to investigate the effect of rapamycin on ASN-mediated ODC induction. Figure 9 shows that rapamycin did not inhibit the induction of ODC activity mediated by ASN, whereas serum-induced enzyme activity was inhibited 85% by the same dose. The failure of rapamycin to block the response to serum entirely can be ascribed to the presence of amino acids in the serum as well as to the growth factors, the effects of which were inhibited. The reason Seidel and Ragan saw only 50% inhibition in response to serum is probably because their studies were conducted in medium that supplies a full complement of amino acids. These results rule out the possibility of the involvement of eIF-4F and new protein synthesis in the induction of ODC activity by ASN and establish that ODC induction by ASN is a distinct process from that of growth factors.

The half-life of ODC activity was 66 min in the presence of both ASN and EGF compared with 46 min with ASN alone (Fig. 6). A 76–84% decrease in induced ODC activity after a 2-h cycloheximide treatment was not accompanied by a decrease in ODC protein (Fig. 8). Thus, in the absence of ongoing protein synthesis, ODC activity induced by either ASN alone or the combination of ASN and EGF declines rapidly.

The decay in ODC activity was only 26% slower in the absence of cycloheximide in cells treated with ASN plus EGF compared with those treated with ASN alone, which was not significant. These observations indicate the involvement of an additional protein factor for the assembly or stabilization of the active conformation of the ODC protein. Protein turnover is low in IEC-6 cells, and ODC protein may be subject to posttranslational control, causing rapid activation-inactivation cycles in response to proliferative stimuli.

ODC exists in both phosphorylated and unphosphorylated forms in RAW 264 cells (30). Phosphorylated ODC was more stable and had a greater catalytic efficiency and higher maximal velocity, demonstrating the requirement of an as yet unidentified protein kinase in the induction of ODC activity. Chen and Canellakis (3) reported that, in mouse N18 neuroblastoma cells, the addition of ASN increased the half-life of ODC from 12.5 to 420 min. The addition of actinomycin D along with ASN had no effect, whereas the addition of cycloheximide with ASN inhibited the induction of ODC activity. In contrast, in our study, the induction of ODC activity was inhibited by both actinomycin D and cycloheximide (Table 1), indicating the necessity of both transcription and translation and suggesting the requirement of de novo protein synthesis.

In summary, we have shown the presence of two synergistic mechanisms for maximal induction of ODC activity. Stimulation by EGF involved eIF-4F, whereas activation of ODC by ASN did not. Although induction of ODC activity did not involve the synthesis of ODC protein, it did require both transcription and translation. These results indicate that a specific inducible factor transcribed and translated in the presence of ASN and EGF is essential for the induction of ODC activity. We suggest that this factor is part of the structural requirement for the formation of the active homodimer of ODC, indicating the mechanism for the posttranslational regulation of the enzyme.

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