The EMBO Journal Vol.18 No.5 pp.1214–1222, 1999

Translocation of ornithine decarboxylase to the surface membrane during cell activation and transformation

Marja Heiskala[1,2,3], Jian Zhang[1], Shin-ichi Hayashi[4], Erkki Hölttä[1] and Leif C.Andersson[1,5]

[1]Department of Pathology, Haartman Institute, and Helsinki University Hospital, University of Helsinki, Haartmaninkatu 3, FIN-00014 Helsinki, Finland, [2]The R.W.Johnson Pharmaceutical Research Institute, 3535 General Atomics Court, Suite 100, San Diego, CA 92121, USA, [4]Department of Biochemistry II, The Jikei University School of Medicine, Minato-ku, Tokyo 105, Japan and [5]Institute for Oncology and Pathology, Karolinska Institute, S-171 76 Stockholm, Sweden

[3]Corresponding author
e-mail: mheiskal@prius.jnj.com

Ornithine decarboxylase (ODC) is highly up-regulated in proliferating and transforming cells. Here we show that upon induction, an initial cytosolic increase of ODC is followed by translocation of a fraction of the enzyme to the surface membrane. ODC membrane translocation is mediated by a p47^{phox} membrane-targeting motif-related sequence, as indicated by reduced ODC activity in the membrane fraction of cells treated with a competing, ODC-derived (amino acids 165–172) peptide, RLSVKFGA, which is homologous to the p47^{phox} membrane-targeting sequence. p47^{phox} membrane translocation is known to be dependent on the phosphorylation of the targeting motif. Analogously, overexpressed ODC.S167A, a mutant ODC lacking the putative phosphorylation site Ser67, is unable to move to the surface membrane. Cells blocked with the RLSVKFGA peptide showed defective transformation, indicating that the motif-mediated translocation of ODC is prerequisite to its biological function. Constitutive targeting of ODC to the membrane using a plasmid encoding the chimeric protein, wild-type ODC with C-terminal linkage to the farnesylation motif of K-ras, caused impaired cytokinesis with an accumulation of polykaryotic cells. Impaired cytokinesis confirms that ODC is involved in mitotic cytoskeletal rearrangement events and pinpoints the importance of relevant membrane targeting to its physiological function.

Keywords: cell activation/membrane targeting/ODC

Introduction

Ornithine decarboxylase (ODC) regulates the rate of polyamine synthesis by catalyzing the generation of putrescine and CO_2 from ornithine. ODC activity and polyamines are connected intimately to cell proliferation and transformation (Tabor and Tabor, 1984; Pegg, 1986, 1988; Heby and Persson, 1990). Quiescent cells display low ODC activity, whereas mitogenic triggering induces a rapid up-regulation of ODC activity. Moreover, the activity of ODC varies during the cell cycle in normal cells. The activity starts to rise in mid-G_1 and peaks in G_2 before the onset of mitosis (Fredlund *et al.*, 1995). Transformed cells, on the other hand, display constitutively elevated ODC activities (Pegg, 1988; Auvinen *et al*., 1992).

We reported previously that the elevated ODC activity found in malignant cells is not merely a consequence of cell transformation, but that ODC activity and/or polyamines are involved in the mechanisms that lead to the transformed phenotype. When rat 2R cells infected with a temperature-sensitive (ts) mutant of v-src were treated with the selective ODC inhibitor, difluoromethylornithine (DFMO), and shifted to the permissive temperature in the absence of exogenous polyamines, cell transformation did not occur. Putrescine restored cell transformation at the permissive temperature. Furthermore, overexpression of normal human ODC in NIH 3T3 cells induced malignant transformation, including the ability to grow as colonies in semi-solid medium and to form rapidly progressing tumors in nude mice (Auvinen *et al.*, 1992, 1997; Moshier *et al.*, 1993; Clifford *et al.*, 1995).

We have found that ODC regulates the level of tyrosine phosphorylation of the Src substrate p130^{cas} (Auvinen *et al*., 1995), a phosphoprotein involved in the formation of signaling complexes at the cytoplasmic side of the plasma membrane in focal adhesion sites (Parsons, 1996). Regulation of phosphorylation of p130^{cas}, together with the suggested association of ODC with transmembrane calcium and/or calmodulin signaling (Ginty and Seidel, 1989) and with activation-mediated membrane translocation of protein kinase C (PKC) (Groblewski *et al.*, 1992), point to the plausibility of ODC also exerting activity at the plasma membrane. However, ODC has been regarded as a cytoplasmic protein (Pegg, 1986). Direct visualization of the subcellular location of ODC has been difficult due to its low concentration and short half-life. To determine its localization, we combined double-sandwich immunostaining of ODC and confocal microscopy with measurements of ODC activity in subcellular fractions. Using this combined approach, we observed that a fraction of ODC translocates from the cytosol to the surface membrane during cell activation and transformation. We also identified a p47^{phox}-related peptide motif, containing a putative serine phosphorylation site that is involved in the physiological membrane translocation of ODC. To study the consequences of constitutive binding of ODC to the plasma membrane, we transfected cells with a chimeric ODC plasmid that contained a 3′ sequence encoding the 17 C-terminal amino acids of K-ras, including the membrane-targeting farnesylation motif.

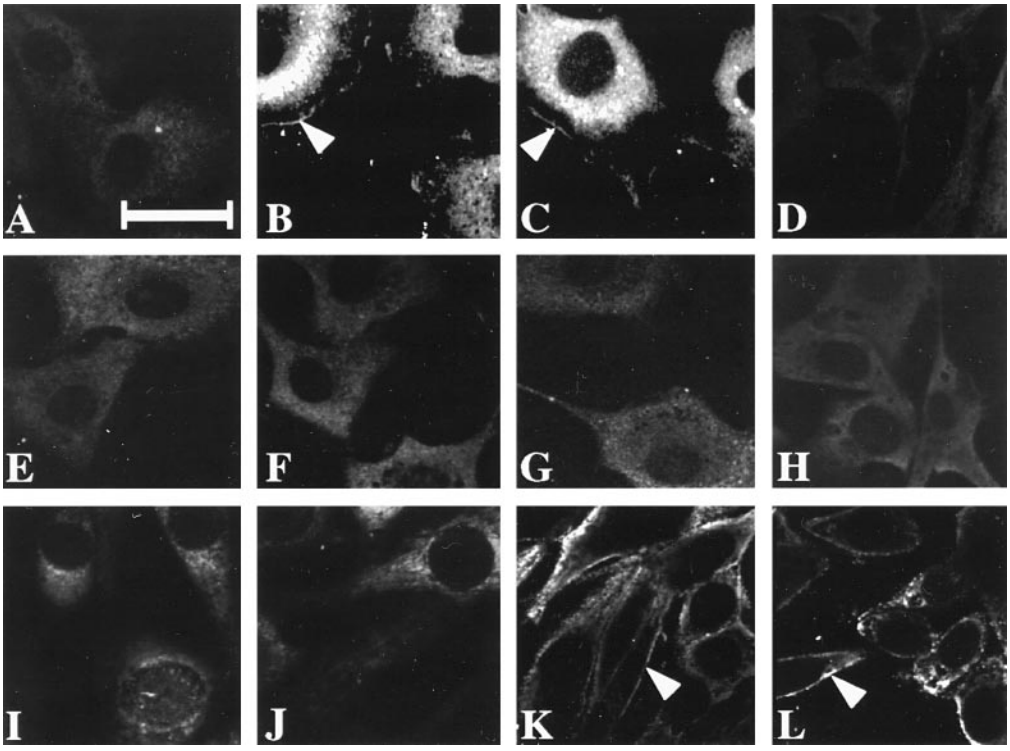

Fig. 1. Fluorescent immunostaining of ODC in quiescent Rat-1 tsRSVLA29 cells (**A**) and in cells activated by 100 nM TPA for 2 (**B**), 4 (**C**) and 24 h (**D**) is shown. As a control, similarly treated cells were stained using monoclonal anti-CD3 (OKT3) as the primary antibody (**E**–**H**). ODC immunostaining in quiescent cells is shown (**I**), as well as cells exposed to the permissive temperature for 1 (**J**), 4 (**K**) and 24 h (**L**). Arrowheads indicate membrane staining. Bar, 25 μm.

Results

Localization of ODC in resting and stimulated Rat-1 tsRSVLA29 cells by immunostaining and confocal microscopy

Rat-1 tsRSVLA29 cells were grown on glass coverslips at the non-permissive temperature (39.5°C) for 2 days and were then induced to up-regulate ODC activity by treatment with the following stimuli: (i) 100 nM 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA); (ii) hypotonic (50% volume H_2O) medium; (iii) dialyzed human AB serum (15%) after 13 h of serum starvation; or (iv) transfer to permissive temperature (35°C) to induce ts-v-src-driven transformation. At selected time points, the cells were fixed, permeabilized and stained for ODC using double-fluorescein isothiocyanate (FITC) labeling. Cells subjected to the first three stimuli displayed a transient membrane staining (Figure 1) that was not observed in the unstimulated control cells. In Rat-1 tsRSVLA29 cells transformed at the permissive temperature (Figure 1), prolonged membrane staining with anti-ODC antibodies was seen.

To substantiate further the membrane translocation of ODC observed by immunofluorescence, we measured ODC activity in the membrane and soluble fractions of activated and control Rat-1 tsRSVLA29 cells. In cells treated with 100 nM TPA for 2, 4 and 24 h, the increase of ODC activity in the soluble fraction peaked at 2 h, then decreased, reaching the control level at 24 h. For these cells, the membrane fraction showed the highest levels of activity at 4 h (Figure 2A). The kinetics of ODC activity were somewhat slower in the soluble fractions of hypotonia-treated cells, where the peak activity in both soluble and membrane fractions was reached at 4 h (Figure 2B). Similar kinetics of ODC activity were encountered in Rat-1 tsRSVLA29 cells subjected to serum after 13 h of starvation (Figure 2C). When the cells were shifted from 39.5°C to the permissive temperature (35°C), activation of ts-v-src induced ODC activity in the soluble fraction within 2–3 h. In the membrane, ODC activity was rising to 24 h concomitantly with the cells acquiring a transformed phenotype (Figures 2D and 1L). In activated cells, a fraction comprising 10–20% of the total elevated ODC activity displayed membrane translocation.

The sequence surrounding Ser167, a membrane-targeting motif in ODC

The cytosolic proteins $p47^{phox}$ and $p67^{phox}$ are components of the NADPH-dependent leukocyte respiratory burst oxidase. These proteins become phosphorylated on serine residues and translocate to the membrane in activated polymorphonuclear neutrophils (Clark *et al*., 1990). Treatment of leukocytes with TPA redistributes a proportion of $p47^{phox}$ to the membrane (Tisch *et al*., 1995). Nauseef *et al*. (1993) found that a putative serine phosphorylation domain corresponding to residues 323–332 of $p47^{phox}$ (**A**YR**R**N**S**V**R**FL) is crucial for the membrane translocation of $p47^{phox}$. It is noticeable that Ser167 of ODC is surrounded by the sequence **A**VC**R**L**S**VK**F**G, which is homologous to the membrane-targeting motif of $p47^{phox}$. In order to test the impact of this motif on ODC function, Rat-1 tsRSVLA29 cells were cultured at the non-permissive temperature for 3 h with either (i) 0.2–2 μg/ml of the synthetic peptide RLSVKFGA linked via a proline to an antennapedia (RQIKIWFQNRRMKWKK) peptide to

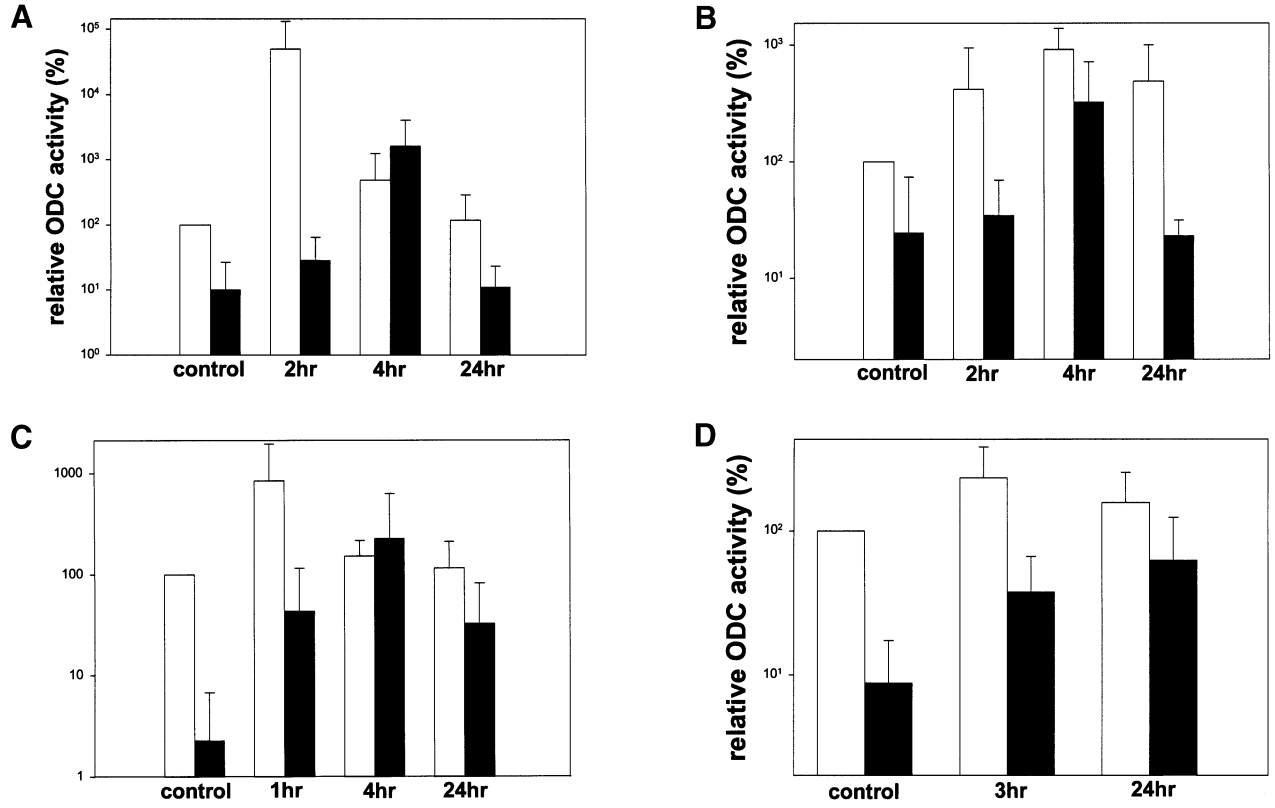

Fig. 2. The relative ODC activity/milligram of protein in Rat-1 tsRSVLA29 cell soluble (open bars) and membrane fractions (filled bars), expressed as mean ± SD of the percentage of the activity of the soluble fraction of quiescent control cells indicated by 100% (3–7 experiments/group). The mode of activation was TPA (**A**), hypotonia (**B**), 15% serum (**C**) and permissive temperature (**D**).

facilitate transmembrane transport (Hall *et al*., 1996), or (ii) 0.2–2 μg/ml of an antennapedia-linked control peptide (ELFHQLACRECHVPL). After 3 h, cells were transferred to the permissive temperature. Incubation with the relevant peptide greatly delayed ts-v-src-driven transformation as compared with untreated control cells or cells treated with the nonsense peptide (Figure 3). In other experiments, the RLSVKFGA peptide without the antennapedia was used. These experiments required higher concentrations of the peptide (100–400 μg) to produce identical results, probably a result of less efficient cellular uptake. Using the same concentrations of the scrambled control peptide (VARGFLESK) had no effect on the level or compartmentalization of ODC activity. Measuring ODC activities from the peptide-treated Rat-1 tsRSVLA29 cells consistently revealed a dose-dependent suppression of the permissive temperature-induced increase in the membrane-associated ODC activity. This suppression did not result from non-specific toxicity since the cells remained responsive as indicated by an undisturbed increase in the overall activity observed in response to the permissive temperature (Figure 4).

Ser167 is required for membrane translocation of ODC

cDNAs encoding wild-type (wt) ODC and mutated ODC lacking the putative phosphorylation site of the phox47-related membrane-targeting motif RLSVKFGA (with a substitution of Ser167 by alanine) were amplified by PCR and cloned into the *Xba*I site of the pCHA expression vector (pCHA-wtODC and pCHA-ODC.S167A). The hemagglutinin (HA)-epitope tag of the pCHA vector enables endogenous ODC to be distinguished from transfected ODC. Neither the HA tag nor the S167A mutation invalidated the catalytic activity of ODC (data not shown).

Upon transfection of Rat-1 tsRSVLA29 cells, overexpression of both wtODC-HA and ODC.S167A-HA increased total ODC activity. The proportion of increased ODC activity induced by the permissive temperature was lower in the membrane fraction of cells expressing ODC.S167A-HA than in cells expressing wtODC-HA. This finding was consistent in four independent experiments (Table I).

In order to follow the localization of overexpressed HA-tagged ODC visually, the wtODC-HA and ODC.S167A-HA fragments were cloned into the *Hin*dIII site of the pLTRpoly vector for higher expression levels. Resting cells transfected with pLTRpoly-wtODC or with pLTRpoly-ODC.S167A displayed mainly a diffuse cytosolic staining; after transfer to the permissive temperature, only cells expressing wtODC-HA showed clear, membrane-associated staining (Figure 5).

Expression of wtODC and ODC.C360A containing the K-ras-2 membrane-targeting motif and the HA epitope

To investigate the functional role of the membrane translocation of ODC that was observed in activated cells, we designed plasmid constructs to constitutively target the expressed protein to the plasma membrane. In order to test whether the decarboxylation activity of overexpressed ODC is required to induce the transformed

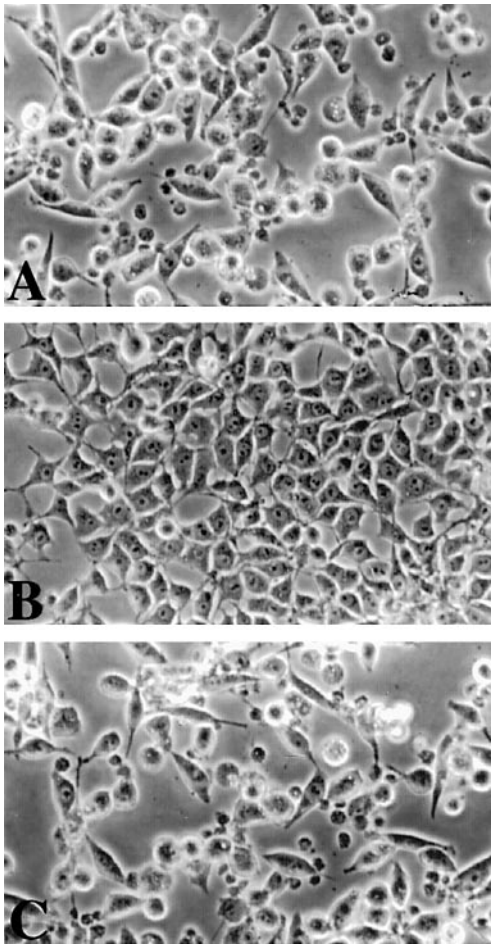

Fig. 3. Rat-1 tsRSVLA29 cells were cultured at the non-permissive temperature (39.5°C) for 2 days to make them quiescent. They were treated subsequently for 3 h either with the solvent [PBS; (**A**)], with 1 μg/ml of the RLSVKFGA (ODC Ser167-surrounding octapeptide, homologous to phox47 membrane-targeting motif)–antennapedia peptide chimera (**B**) or with 1 μg/ml of the control peptide–antennapedia chimera (**C**), whereafter they were transferred to the permissive temperature (35°C) to induce v-src-driven transformation. The cells were photographed 5 h later.

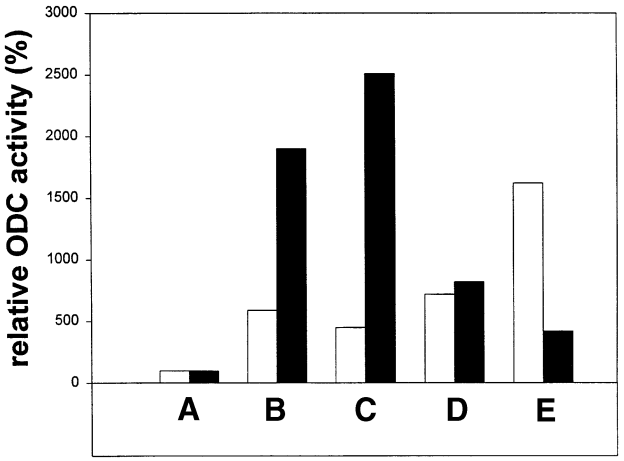

Fig. 4. Rat-1 tsRSVLA29 cells were treated with the solvent [PBS; (**A**) and (**B**)], with 400 μg/ml of control peptide VARGFLSK (**C**) or with 100 and 400 μg/ml of ODC Ser167-surrounding peptide RLSVKFGA [(**D**) and (**E**), respectively]. After a 3 h pre-treatment with PBS or peptides at the non-permissive temperature (39.5°C), the control cells (A) were maintained at 39.5°C, whereas the other cultures (B–E) were transferred to the permissive temperature (35.0°C) for 5 h before harvesting. The relative ODC activities are expressed as percentage of control (soluble cell fraction of the untreated cells at non-permissive temperature, counts/mg of protein). Open bars indicate ODC activity in the soluble fraction, and filled bars indicate the membrane fraction. (One representative experiment out of three is shown.)

Table I. Distribution of ODC activity/mg protein in Rat-1 tsRSVLA29 cells after 12 h at the permissive temperature

	Experiment 1		Experiment 2	
	m	c	m	cc
Wt	4.2	6.1	5.9	11.3
EV	ND	ND	7.7	14.7
ODC	19.8	38.7	23.0	54.1
ODC.S167A	5.5	56.2	11.8	91.1

Wt, untransfected control Rat-1 tsRSVLA29 cells; EV, cells transfected with the empty vector; ODC, cells overexpressing wtODC; ODC.S176A, cells overexpressing mutant ODC; m, membrane fraction; c, cytosolic fraction; ND, not done.

phenotype or whether the mere overexpression and/or membrane targeting of the protein is sufficient, Cys360 was mutated to alanine, an alteration known to abolish >90% of the enzymatic activity of ODC (Coleman, 1993). NIH 3T3 cells were transfected with the following plasmids: pNHA, pCHA, pCHA-ODC, pNHA-ODC-K-ras-2 adhesion sequence, pCHA-ODC.C360A and pNHA-ODC.C360A-K-ras-2 adhesion sequence. After G418 selection, the transfected cells were stained for the HA epitope. Positive immunofluorescence was evident in the membranes of all the cells transfected with a plasmid encoding the ODC-K-ras adhesion sequence, whereas morphological changes only occurred when a construct encoding enzymatically active wtODC was used (Figure 6). In early passages, these cells showed a high frequency of polykaryosis (Figures 6 and 7) and could not be recovered for continuous cultures. The cells that remained in prolonged cultures displayed a round, flattened shape with multiple cellular protrusions (Figure 7). Cells transfected with the pNHA-ODC-K-ras-2 plasmid showed a 40-fold elevated ODC activity in the membrane fraction as compared with the control; the cells transfected with the pCHA-ODC plasmid had a 5-fold elevated enzyme activity in both the cytosolic and the membrane fractions (Figure 8). These elevated activities suggest that the high, persistent ODC activity in the plasma membrane was related causally to the morphological changes. Assays for ODC enzyme activity also revealed that the cells transfected with the pNHA-ODC.C360A plasmid did not display increased activity but rather marginally lower ODC activities than seen in control cells, indicating that ODC.C360A may have a dominant-negative impact (Figure 8).

These findings demonstrate that ODC-related alterations in cell morphology and growth behavior are dependent on intact ODC enzyme activity. These findings also show that for the physiological function of ODC, membrane targeting using the phosphorylation-regulated, $p47^{phox}$-related motif is mandatory. Constitutive (irrelevant) membrane targeting of the active enzyme leads to defective cytokinesis.

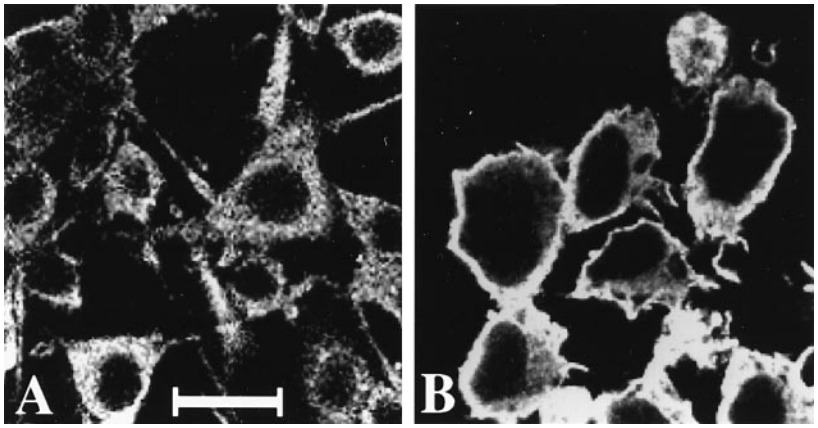

Fig. 5. Rat-1 tsRSVLA29 cells overexpressing ODC.S167A-HA (**A**) and wtODC-HA (**B**) were transferred to the permissive temperature for 12 h, fixed and immunostained for ODC-HA. Extensive membrane staining is only seen in cells expressing wtODC. Bar, 25 μm.

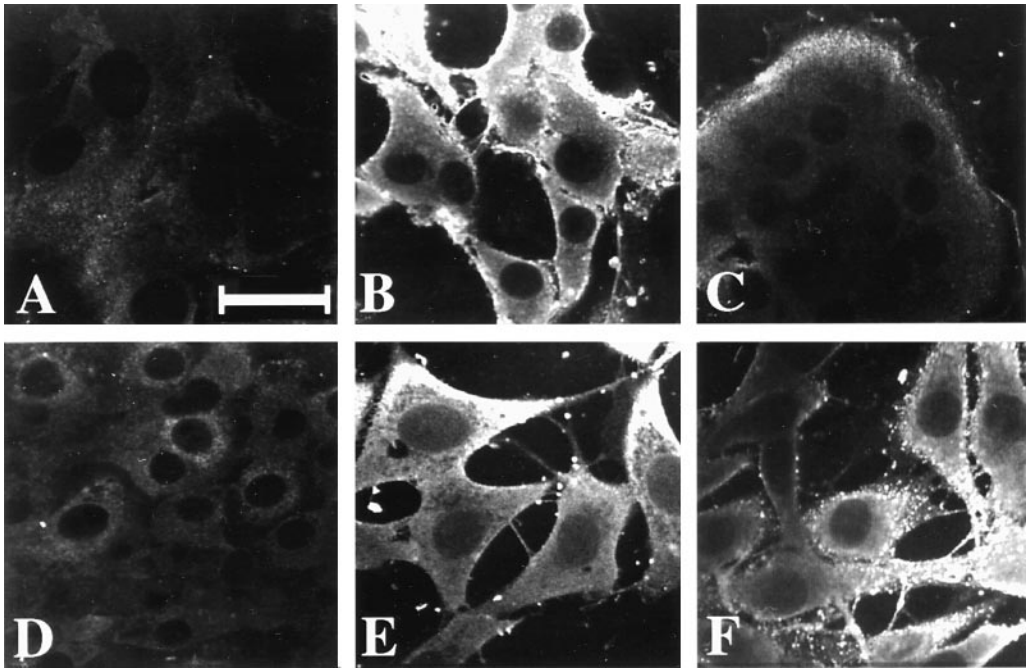

Fig. 6. NIH 3T3 cells, transfected with empty pCHA (**A**) and pNHA vectors (**D**), pCHA-ODC (**B**), pNHA-ODC-K-ras-2 adhesion tag (**C**), pCHA-ODC.C360A (**E**) and pNHA-ODC.C360A-Ki-ras-2 adhesion tag (**F**), were immunostained using monoclonal anti-HA as the first antibody. Bar, 25 μm.

Discussion

The cellular level of ODC is strictly feedback controlled. Elevated cellular concentrations of polyamines induce up-regulated expression of antizyme, an ODC-inhibitory protein, that binds ODC and induces its rapid degradation by the 26S proteasome complex independently of ubiquitination (Tokunaga *et al*., 1994). This degradation leads to a fast turnover of ODC, which is, in fact, one of the most short-lived enzymes in mammalian cells, having a half-life of 10–20 min. Given these features, the cellular concentration of ODC is very low, estimated to comprise 0.0003–0.01% of total cellular proteins (Heby and Persson, 1990). Therefore, visualizing the subcellular distribution of ODC has been difficult.

Previously, ODC was regarded mainly as a cytosolic protein (Pegg, 1986). To follow the distribution of ODC in resting and in activated and/or transformed cells, we combined confocal microscopy with the following five-step sandwich immunostaining treatment: (i) monoclonal anti-ODC or anti-HA antibodies; (ii) biotinylated anti-mouse antibodies; (iii) FITC/avidin; (iv) biotinylated anti-avidin; and (v) FITC/avidin. In response to cell activation, regardless of the mode (TPA, hypotonic medium or serum after 13 h of starvation), as well as in response to transformation (induced at permissive temperature by ts-v-src), we consistently found translocation of immunoreactive ODC to the plasma membrane. The overexpressed ODC product was monitored by the monoclonal antibody anti-HA 12CA5 against the HA epitope and was found to display the same membrane location as endogenous ODC upon stimulation. Using core-labeled [^{3}H]ornithine as the substrate, ODC enzyme assays of cell fractions revealed an initial rise in soluble fraction activity followed by translocation of a proportion of the ODC activity to the membrane fraction. These findings demonstrate that ODC behaves analogously to

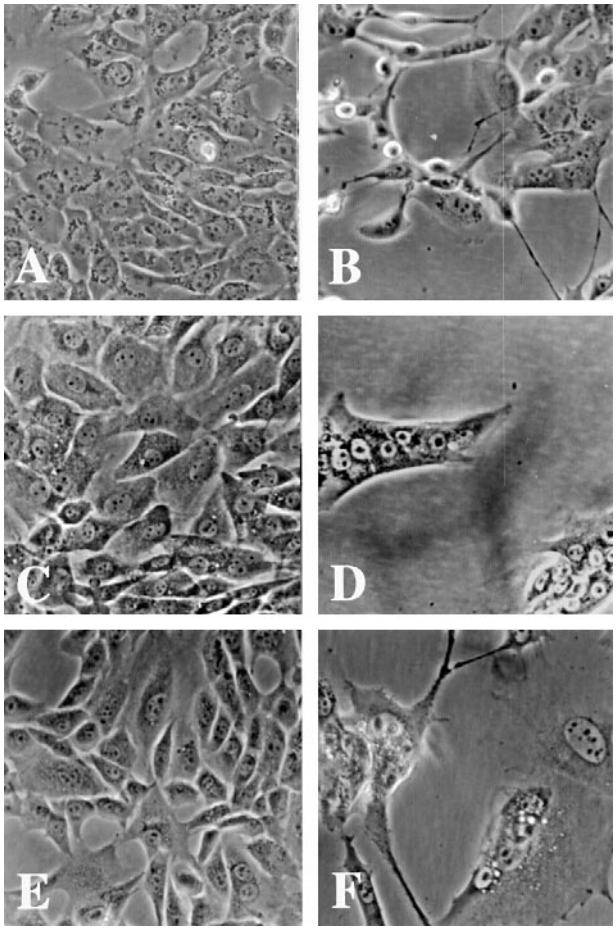

Fig. 7. NIH 3T3 cells transfected with pCHA-ODC.C360A (**A**), pCHA-ODC (**B**), pNHA-ODC.C360A-K-ras-2 adhesion tag, early passages (**C**), pNHA-ODC-K-ras-2 adhesion tag, early passages (**D**), pNHA-ODC.C360A-K-ras-2 adhesion tag, later passages (**E**) and pNHA-ODC-K-ras-2 adhesion tag, later passages (**F**).

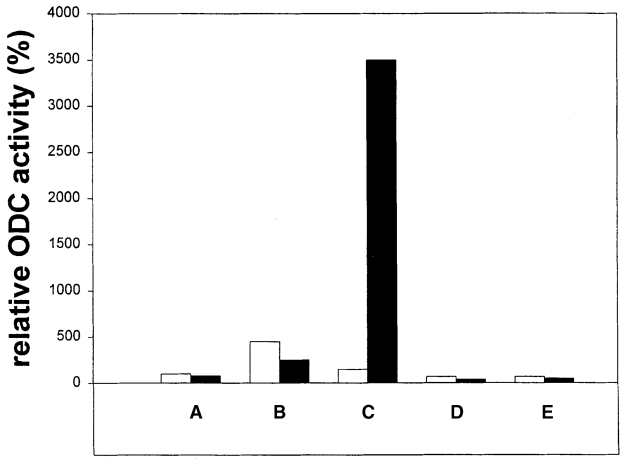

Fig. 8. ODC activity in NIH 3T3 cells, transfected with an empty pNHA vector (**A**), pCHA-ODC (**B**), pNHA-ODC-Ki-ras-2 adhesion tag (**C**), pCHA-ODC.C360A (**D**) and pNHA-ODC.C360A-Ki-ras-2 adhesion tag (**E**), expressed as a percentage of control (the activity, counts/mg, of the soluble fraction of cells transfected with empty pNHA vector). Open bars indicate ODC activity in the soluble fraction, and filled bars indicate ODC activity in the membrane fraction. (One representative experiment out of three is shown.)

several other signaling molecules (i.e. PKC, GTP-binding proteins of the Rho–Rac family, c-Raf and the SH2 domain-containing Shc) which, upon cell activation, are recruited transiently close to the plasma membrane (Bokoch *et al*., 1994; Lankester *et al*., 1994; Stokoe *et al*., 1994; Matowe and Ginsberg, 1995).

When investigating the mechanism of ODC membrane translocation, we found that intact catalytic activity of ODC is not required, since the HA-tagged, C360A-mutated ODC displayed normal membrane association in activated cells (data not shown). Treating Rat-1 tsRSVLA29 cells with an antennapedia sequence-carried peptide, representing the eight amino acids around Ser167 of ODC and having homology to the previously described membrane-targeting motif of $p47^{phox}$ (Nauseef *et al*., 1993), dramatically delayed ts-v-src-induced transformation and suppressed the permissive temperature-induced increase in ODC activity in the membrane compartment. The concomitant rise in cytosolic ODC remained intact, indicating that the cells were responsive and suggesting that the motif surrounding Ser167 is important for ODC membrane translocation, which, in turn, is crucial for cell transformation. A direct demonstration of phosphorylation of Ser167 is technically difficult due to the low amount of ODC protein present, even after overexpression. Instead, to substantiate further the role of the $p47^{phox}$-related motif, we mutated Ser167 to alanine. This single amino acid substitution abolished the membrane translocation of over-expressed ODC upon cell activation, indicating that phosphorylation of Ser167 is the prerequisite of ODC membrane translocation and confirming that the phox47-related Ser167-surrounding octamere is involved in ODC membrane association. While v-src-driven transformation induced an extended localization of ODC to the plasma membrane, cells activated with hypotonic medium, TPA or serum displayed a transient rise in membrane-associated ODC activity. To study the impact of constitutively membrane-associated ODC, we constructed plasmids to express chimeric ODC containing the C-terminal farnesylation motif of K-ras. We used vectors with the cytomegalovirus (CMV) promoter, which yields modest levels of protein in rodent cells. Upon transfection into NIH 3T3 cells, these plasmids still had a dramatic impact on cell morphology. At an early phase of G418 selection, multinucleated, large, flattened cells with extended cytoplasmic protrusions were seen. The generation of polykaryosis suggests that constitutive overexpression of ODC at the membrane interferes with cytokinesis. Defective cytokinesis may be caused by constitutively high ODC activity in the membrane and/or may be due to the non-physiological mode of targeting, such as use of the Kras farnesylation motif instead of the $p47^{phox}$-related phosphorylation-regulated membrane targeting motif, i.e. targeting of ODC to the membrane lipid layer instead of to the membrane cytoskeleton (Woodman *et al*., 1991; El-Benna *et al*., 1994). Since reorganization of the membrane actin skeleton is the prerequisite for cytokinesis and acquisition of a transformed phenotype, it appears plausible that the membrane translocation of ODC in activated cells indicates an association with the membrane actin skeleton rather than with the lipid membrane.

The ultimate molecular mechanism(s) by which ODC contributes to the regulation of cell transformation remains

to be elucidated. The disassembly of the organized cytoskeleton that takes place during malignant transformation apparently implies 'misuse' of the genetic program that physiologically regulates initiation of mitosis. Thus, it is tempting to speculate that the steeply increased ODC activity found in G_2 in normal cells and the high ODC activity found in transformed cells represent a similar molecular role for ODC in both events. Indeed, we recently found that the ODC activity in the cell membranes of synchronized Rat-2 fibroblasts is low in S-phase cells, whereas in the membranes of G_2- and/or M-phase cells an increase of >10-fold increase is seen (M.Heiskala and L.C.Andersson, in preparation). Understanding the regulation of the obviously essential phosphorylation of the $p47^{phox}$ related membrane targeting motif is important for a comprehensive view of how ODC eventually triggers the cellular machinery, which leads to the reorganization of the cytoskeleton. Phox47 has been reported to be phosphorylated in numerous serines by PKC, PKA and $p65^{pak}$/PAK2. These kinases probably also act on the 323–332 residue motif, which is crucial for phox67 and membrane binding (Knaus *et al*., 1995; De Leo *et al*., 1996; El-Benna *et al*., 1996). It will be of interest to identify the kinase(s) that phosphorylate ODC. Not only do these kinases play a central role in controlling ODC-mediated cellular events, but they are also potential targets for potential drugs for treating proliferative diseases.

Materials and methods

Cells and reagents

The ts RSV-infected rat fibroblast cell line, Rat-1 tsRSVLA29, was obtained from Dr J.Wyke (Wyke *et al*., 1980). Before experiments, cells were cultured at the non-permissive temperature (39.5°C) for 48 h in RPMI-1640 medium supplemented with 10% fetal calf serum, glutamine and antibiotics. Cells were grown until 80–90% confluent. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum, glutamine and antibiotics. TPA was obtained from Sigma Chemical Co. (St Louis, MO).

Plasmid constructions and transfections

Human ODC cDNA (Hickok *et al*., 1987) was used as the template for PCR amplification, and the oligonucleotides 5′-GCTCTAGAAACAACTTTGGTAAT-3′ (5′ primer) and 3′-GCTCTAGAAAGCTTCACATTAATACTA-5′ (3′ primer) were used to amplify cDNA encoding ODC. The ODC cDNA fragment was flanked by *Xba*I sites at both ends and a *Hin*dIII site at the 3′ end proximal from the *Xba*I site. Following amplification, the PCR product was isolated and cloned into the pCHA vector. The pCHA (C-terminal tagging of the protein product) and pNHA (N-terminal tagging of the protein product) vectors are designed for expressing HA epitope-tagged proteins in mammalian cells. The plasmids are composite constructs derived from the 5.4 kb Rc/CMV expression vector (Invitrogen, San Diego, CA), which retains features such as the CMV promoter, a high-copy ColE1 origin, the SP6 and T7 promoters, the neomycin and ampicillin resistance genes and the M13 origin for mutagenesis (Pati, 1992). The insert was sequenced, and the plasmid was used for transfections.

To express ODC containing a membrane-targeting motif, we prepared a cDNA fragment encoding a c-K-ras-derived, C-terminal membrane adhesion tag. The pUC 13/c-Ki-ras-2 (human, ATCC; McCoy *et al*., 1984) plasmid was used as a template, and the 5′ and 3′ primers, 5′-CGGGGTACCAGCAAAGATGGTAAA-3′ and 3′-CGCGGATCCTTACATAATTACACA-5′, were used to amplify by PCR a cDNA fragment encoding the 17 C-terminal amino acids of c-K-ras-2. The cDNA fragment was flanked by a 5′ *Kpn*I site and a 3′ *Bam*HI site. Following amplifications, the fragment was cloned into the pcDNA3 vector (Invitrogen) and sequenced. The HA-ODC cDNA fragment from pNHA-ODC was cloned into the *Hin*dIII site of the pcDNA3/c-K-ras-2 adhesion sequence. The ODC-K-ras-2 adhesion tag from pcDNA3 was then cloned into the *Xba*I site of the pNHA vector and used for transfections.

To assess the importance of the enzyme activity of ODC, we designed an enzymatically inactive ODC derivative (ODC.C360A). The point mutation was introduced by PCR using human ODC cDNA as a template and the internal primers 5′-GGGACCAACA**GC**TGATGGCCTC-GATC-3′ and 3′-GAGGCCATCA**GC**TGTTGGTCCCCATA-5′ together with the described outer primers to create a template for cDNA encoding for ODC.C360A.

Similarly, to change the Ser167 in the $p47^{phox}$-homologous motif RLSVKFGA to alanine, a mutated cDNA encoding for ODC.S167A was made using inner primers 5′-AGTCTGTCGTCTC**GC**TGTG-AAATTCGG-3′ and 3′-CCGAATTTCACA**GC**GAGACGACAGA-5′ to create a template. The end products were sequenced, and the presence of the changes of codon 360 TGT into GCT and the codon 167 AGT to GCT were verified. The routine PCR amplifications were made using Deep Vent DNA polymerase (New England Biolabs, Beverly, MA) according to the manufacturer's instructions, whereas the point mutation-containing cDNAs were amplified by PCR using *Taq* DNA polymerase (New England Biolabs), and treated with Deep Vent in the presence of dNTPs for 20 min at 37°C before use as templates.

NIH 3T3 cells were transfected with empty vectors pCHA and pNHA, pCHA-ODC, pNHA-ODC-K-ras-2 adhesion tag, pCHA-ODC.C360A and pNHA-ODC.C360A-K-ras-2 adhesion tag. Rat-1 tsRSVLA29 cells were transfected with these plasmids and with the pCHA-ODC.S167A plasmid. For higher protein expression levels, the wtODC-HA and ODC.S167A-HA fragments were subcloned into the *Hin*dIII site of the pLTRpoly vector containing the U3 region of the long terminal repeat of Moloney murine leukemia virus and the SV40 splicing and poly-adenylation signals (Mäkelä *et al*., 1992). To follow the localization of overexpressed wtODC and ODC.S167A by fluorescence microscopy, Rat-1 tsRSVLA29 cells were transfected with the pLTRpoly-ODC and pLTRpoly-ODC.S167A plasmids and, for neomycin selection, co-transfected with the pSV2neo plasmid (ATCC 37419; Southern *et al*., 1982).

Cells were transfected using 5 μg of the plasmid per 10^5 cells using the lipofectamine method according to the manufacturer's instructions (Lipofectamine Reagent, Life Technologies Inc., Gaithersburg, MD).

Induction of ODC response

ODC activity was induced in Rat-1 tsRSVLA29 cells kept at the non-permissive temperature (39.5°C) by treatment with the following stimuli: (i) 100 nM TPA; (ii) 1 vol. of H_2O to create 50% hypotonicity; or (iii) 15% human AB serum (Finnish Red Cross) added to cells after 13 h of serum starvation. The serum was dialyzed extensively against phosphate-buffered saline (PBS), PBS supplemented with 0.01 M $MgCl_2$, and 1 M $NaCl_2$, to deplete polyamines. The cells were harvested after 1, 2, 4 and 24 h incubations. Control cells were treated with: (i) the corresponding solvent only; (ii) normal culture medium; or (iii) maintained in serum-free cultures. In order to study the ODC response induced by ts-v-src-mediated transformation, Rat-1 tsRSVLA29 cells cultivated at the non-permissive temperature (39.5°C) were shifted to the permissive temperature (35°C).

Synthetic peptides

The sequence surrounding Ser167 of ODC, which is homologous to the $p47^{phox}$ membrane-targeting site, and a control peptide consisting of the same amino acids randomly arranged, were synthesized by the Haartman Institute Peptide Laboratory, University of Helsinki, using the solid-phase method on an Applied Biosystems 433A Peptide Synthesizer (Foster City, CA). Rat-1 tsRSVLA29 cells were grown at the non-permissive temperature in medium supplemented with dialyzed human AB serum (Finnish Red Cross, 2%) for 48 h, treated for 3 h with a fusion peptide (0.2–2.0 μg/ml) consisting of RLSVKFGA (corresponding to amino acids 165–172 of ODC) linked via a proline residue to an antennapedia peptide (RQIKIWFQNRRMKWKK**P**RLSVKFGA) that promotes an active cellular intake (Hall *et al*., 1996). Thereafter, the cultures were transferred to the permissive temperature for 5 h, and the cells were fixed for analysis. A 15 amino acid nonsense sequence linked to the antennapedia peptide (RQIKIWFQNRRMKWKKELFHQLAC-RECHVPL) was used as a control. In some experiments, the ODC Ser167-surrounding peptide RLSVKFGA and the control peptide VARGHLSK without the antennapedia (100–500 μg/ml) were used.

Lysis and fractionation of cellular components

Cells were harvested on ice, washed once with cold PBS, pelleted and suspended in hypotonic buffer containing 25 mM Tris–HCl pH 8.0,

1 mM $MgCl_2$, 1 μM $CaCl_2$, 0.1 mM dithiothreitol (DTT), 0.05% NP-40 (to stabilize ODC) and leupeptin/aprotinin. After 10 min on ice, the samples were centrifuged for 3 min at 600 *g* to remove intact cells and nuclei, and the supernatants were collected. Cytosol and membrane fractions were isolated by a Beckman airfuge (Beckman Instruments Inc., Palo Alto, CA), 15 min at 30 p.s.i. The membrane pellets were washed once with buffer, resuspended and briefly sonicated. The protein concentrations of the samples were measured by Bio-Rad microassay (Bio-Rad Laboratories, Hercules, CA).

Ornithine decarboxylase assay

The ODC activity of the samples was measured according the method of Djurhuus (1981). Briefly, 100 μl of sample was mixed with 15 μl of the following reaction mixture: 5 mM DTT, 20 μM ornithine, 50 μM pyridoxal phosphate and 100 μM EDTA. A 1 μCi aliquot of L-[2,3-^{3}H]ornithine (Dupont NEN, Wilmington, DE) was added and, after a 1 h incubation at 37°C, the mixture was spotted on P81 phosphocellulose filter papers (Whatman International Ltd, Maidstone, UK). Filter papers were then washed with 0.1 M ammonium hydroxide, dried and counted for the radioactivity in a Wallac 1410 liquid scintillation counter (Turku, Finland). The relative ODC activity was calculated as counts/mg of protein.

Immunostaining and confocal microscopy

For immunostaining, Rat-tsRSVLA29 cells were grown on gelatin-coated glass coverslips, washed once with PBS, fixed with cold 3.5% paraformaldehyde and permeabilized with 0.2% NP-40 in PBS. A combination of two monoclonal anti-ODC antibodies [HO 101, IgG1/κ and HO 202, the latter as yet uncharacterized (Matsufuji *et al*., 1984; Nishiyama *et al*., 1988)], and the monoclonal antibody to the HA epitope (12CA5, Boehringer Mannheim, Indianapolis, IN) were used as first antibodies (diluted 1:40 and incubated overnight at 4°C). After washing with PBS/0.05% NP-40, biotinylated anti-mouse IgG (Sigma), FITC/avidin, biotinylated anti-avidin and FITC/avidin (Vector Laboratories, Burlingame, CA) were applied. The cells were inspected with a confocal microscope (Zeiss LSM 410 invert, Oberkochen, Germany). Anti-CD3 (OKT3) was used as a control first antibody.

Acknowledgements

We thank Ms Hannele Laaksonen for her technical help. This work has been supported by the Finnish Academy of Sciences, the Sigrid Juselius Foundation, the Finnish Life and Pension Insurance Companies, the Finnish Cancer Society, the Stockholm Cancer Society and the University of Helsinki.

References

Auvinen,M., Paasinen,A., Andersson,L.C. and Hölttä,E. (1992) Ornithine decarboxylase activity is critical for cell transformation. *Nature*, **360**, 355–358.

Auvinen,M., Paasinen-Sohns,A., Hirai,H., Andersson,L.C. and Hölttä,E. (1995) Ornithine decarboxylase- and ras-induced cell transformations: reversal by protein tyrosine kinase inhibitors and role of pp130^{cas}. *Mol. Cell. Biol.*, **15**, 6513–6525.

Auvinen,M., Laine,A., Paasinen-Sohns,A., Kangas,A., Kangas,L., Saksela,O., Andersson,L.C. and Hölttä,E. (1997) Human ornithine decarboxylase-overproducing NIH3T3 cells induce rapidly growing, highly vascularized tumors in nude mice. *Cancer Res.*, **57**, 3016–3025.

Bokoch,G.M., Bohl,B.P. and Chuang,T.H. (1994) Guanine nucleotide exchange regulates membrane translocation of Rac/Rho GTP-binding proteins. *J. Biol. Chem.*, **269**, 31674–31679.

Clark,R.A., Volpp,B.D., Leidal,K.G. and Nauseef,W.M. (1990) Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. *J. Clin. Invest.*, **85**, 714–721.

Clifford,A., Morgan,D., Yuspa,S., Soler,A.P. and Gilmour,V. (1995) Role of ornithine decarboxylase in epidermal tumorigenesis. *Cancer Res.*, **55**, 1680–1686.

Coleman,C.S., Stanley,B.A. and Pegg,A.E. (1993) Effect of mutations at active site residues on the activity of ornithine decarboxylase and its inhibition by active site-directed irreversible inhibitors. *J. Biol. Chem.*, **268**, 24572–24579.

De Leo,F.R., Ulman,K.V., Davis,A.R., Jutila,K.L. and Quinn,M.T. (1996) Assembly of the human neutrophil NADPH oxidase involves binding of p67^{phox} and flavocytochrome b to a common functional domain in p47^{phox}. *J. Biol. Chem.*, **271**, 17013–17020.

Djurhuus,R. (1981) Ornithine decarboxylase (EC 4.1.1.17) assay based upon the retention of putrescine by a strong cation-exchange paper. *Anal. Biochem.*, **113**, 325–355.

El-Benna,J., Rued,J.M. and Babior,B.M. (1994) Cytosolic guanine nucleotide-binding protein Rac2 operates *in vivo* as a component of the neutrophil respiratory burst oxidase. Transfer of Rac2 and the cytosolic oxidase components p47^{phox} and p67^{phox} to the submembranous actin cytoskeleton during oxidase activation. *J. Biol. Chem.*, **269**, 6729–6734.

El-Benna,J., Faust,L.P., Johnson,L. and Babior,B.M. (1996) Phosphorylation of the respiratory burst oxidase subunit p47^{phox} as determined by two-dimensional phosphopeptide mapping. *J. Biol. Chem.*, **271**, 6374–6378.

Fredlund,J.O., Johansson,M.C., Dahlberg,E. and Oredsson,T.M. (1995) Ornithine decarboxylase and *S*-adenosylmethionine decarboxylase expression during the cell cycle of Chinese hamster ovary cells. *Exp. Cell Res.*, **216**, 86–92.

Ginty,D.D. and Seidel,E.R. (1989) Polyamine-dependent growth and calmodulin-regulated induction of ornithine decarboxylase. *Am. J. Physiol.*, **256**, G342–G348.

Groblewski,G.E., Ways,D.K. and Seidel,E.R. (1992) Protein kinase C regulation of IEC-6 cell ornithine decarboxylase. *Am. J. Physiol.*, **263**, G742–G749.

Hall,H., Williams,E.J., Moore,S.E., Walsh,F.S., Prochiantz,A. and Doherty,P. (1996) Inhibition of FGF-stimulated phosphatidylinositol hydrolysis and neurite outgrowth by a cell-membrane permeable phosphopeptide. *Curr. Biol.*, **5**, 580–587.

Heby,O. (1981) Role of polyamines in the control of cell proliferation and differentiation. *Differentiation*, **19**, 1–20.

Hickok,N.J., Seppänen,P.J., Gunsalus,G.L. and Jänne,O.A. (1987) Complete amino acid sequence of human ornithine decarboxylase deduced from complementary DNA. *DNA*, **6**, 179–187.

Knaus,U.G., Morris,S., Dong,H.-J., Chernoff,J. and Bokoch,G.M. (1995) Regulation of human leukocyte p12-activated kinases through G protein-coupled receptors. *Science*, **269**, 221–223.

Lankester,A.C., van Schijndel,G.M., Rood,P.M., Verhoeven,A.J. and van Lier,R.A. (1994) B cell antigen receptor cross-linking induces tyrosine phosphorylation and membrane translocation of a multimeric Sch complex that is augmented by CD19 co-ligation. *Eur. J. Immunol.*, **24**, 2818–2825.

Mäkelä,T.P., Partanen,J., Schwab,M. and Alitalo,K. (1992) Plasmid pLTRpoly: a versatile high-efficiency mammalian expression vector. *Gene*, **118**, 293–294.

Matowe,W.C. and Ginsberg,J. (1995) Increased membrane-bound protein kinase C in porcine thyroid cells following TSH exposure. *Clin. Invest. Med.*, **18**, 186–192.

Matsufuji,S., Fujita,K., Kameji,T., Kanamoto,R., Murakami,Y. and Hayashi,S. (1984) A monoclonal antibody to rat liver ornithine decarboxylase. *J. Biochem.*, **96**, 1525–1530.

McCoy,M., Bergmann,C.I. and Weinberg,R.A. (1984) Human colon carcinoma Ki-ras2 oncogene and its corresponding proto-oncogene. *Mol. Cell. Biol.*, **4**, 1577–1582.

Moshier,J.A., Dosescu,J., Skunca,M. and Luk,G.D. (1993) Transformation of NIH/3T3 cells by ornithine decarboxylase overexpression. *Cancer Res.*, **53**, 2618–2622.

Nauseef,W.M., McCormick,S., Renee,J., Leidal,K.G. and Clark,R.A. (1993) Functional domain in an arginine-rich carboxyl-terminal region of p47phox. *J. Biol. Chem.*, **268**, 23646–23651.

Nishiyama,M., Matsufuji,S., Kanamoto,R., Takano,M., Murakami,Y. and Shin-ichi,H. (1988) Two-step purification of mouse kidney ornithine decarboxylase. *Prep. Biochem.*, **18**, 227–238.

Parsons,J.T. (1996) Integrin-mediated signalling: regulation by protein tyrosine kinases and small GTP-binding proteins. *Curr. Opin. Cell Biol.*, **8**, 146–152.

Pati,U.K. (1992) Novel vectors for expression of cDNA encoding epitope-tagged proteins in mammalian cells. *Gene*, **114**, 285–288.

Pegg,A.E. (1986) Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.*, **234**, 249–262.

Pegg,A.E. (1988) Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.*, **48**, 759–774.

Southern,P.J. and Berg,P. (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.*, **1**, 237–341.

Stokoe,D., Macdonald,S.G., Cadwallader,K., Symens,M. and Hancock,J.F. (1994) Activation of Raf as a result of recruitment to the plasma membrane. *Science*, **264**, 1463–1467.

Tabor,C.W. and Tabor,H. (1984) Polyamines. *Annu. Rev. Biochem.*, **53**, 749–790.

Tokunaga,F., Goto,T., Koide,T., Murakami,Y., Hayashi,S., Tamura,T., Tanaka,K. and Ichihara,A. (1994) ATP- and antizyme-dependent endoproteolysis of ornithine decarboxylase to oligopeptides by the 26S proteasome. *J. Biol. Chem.*, **269**, 17382–17385.

Tisch,D., Sharoni,Y., Danilenko,M. and Aviram,I. (1995) The assembly of neutrophil NADPH oxidase: effects of mastoparan and its synthetic analogues. *Biochem. J.*, **310**, 715–719.

Woodman,R.C., Ruedin,J.M., Jesaitis,A.J., Okamura,N., Quinn,M.T., Smith,R.M., Curnutte,J.T. and Babior,B.M. (1991) Respiratory burst oxidase-related polypeptides are associated with the cytoskeleton of human neutrophils. *J. Clin. Invest.*, **87**, 1345–1351.

Wyke,J.A., Beamand,J.A. and Varmus,H.E. (1980) Factors affecting phenotypic reversion of rat cells transformed by avian sarcoma virus. *Cold Spring Harbor Symp. Quant. Biol.*, **44**, 1065–1075.

Received September 21, 1998; revised December 30, 1998; accepted January 7, 1999