Ornithine Decarboxylase Induction in Transformation by H-Ras and RhoA

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ABSTRACT

The objective of these studies has been to develop a better understanding of the role of ornithine decarboxylase (ODC) during the neoplastic process, and to determine whether induction of ODC is a necessary component in the action of the ras oncogene. Specifically, we have studied the role of ODC overexpression in signaling pathways mediated by Raf or RhoA. Cells transformed by ras are known to have constitutively high levels of ODC activity that correlate with oncogenic transformation. To determine which pathways downstream of Ras contribute to the regulation of ODC activity, NIH-3T3 cells were transfected with plasmids coding for activated mutants of either H-Ras or RhoA, or oncogenic v-Raf. There was a good correlation between increasing ODC specific activity and change in morphology from normal to transformed in the v-Raf, HRas(61L), and RhoA(63L) clones. Increasing ODC activity also correlated positively with the ability to grow in soft agar in both the H-Ras- and RhoA-expressing cells. In stable transfections, coexpression of the ODC dominant negative mutant K69A/C360A with either HRas(61L) or RhoA(63L) both inhibited intracellular ODC activity and caused a reversal of the transformed phenotype, as measured by a dramatic reduction in the ability of these cells to grow in soft agar and form foci on a monolayer. These results suggest strongly that ODC induction is necessary for transformation by oncogenic Ras. In contrast, expression of K69A/C360A had no effect on the ability of v-Raf-transformed cells to grow in soft agar, although intracellular ODC levels were inhibited. When grown on a monolayer, these cells also maintained their transformed appearance. Furthermore, expression of the ODC dominant negative mutant did not affect the phosphorylation of mitogen-activated protein kinase in v-Raf-transformed cells. These experiments provide strong support for the concept that transformation by activated ras is accompanied by an induction of ODC. The results using RhoA(63L) and v-Raf suggest that this increase in ODC activity is mediated at least in part through a Raf/mitogen-activated protein kinase independent pathway.

INTRODUCTION

A growing body of information is available that suggests that overexpression of ODC plays an important role in tumor growth and development. Overexpression of ODC can lead to cellular transformation in vitro and to increased epithelial tumor invasiveness in vivo. In the mouse skin carcinogenesis model, ODC activity is transiently induced in response to tumor promotion, and is constitutively induced in the resulting papillomas, along with abnormally high polyamine levels. Prevention of this rise in ODC by specific irreversible inhibitors such as DFMO reduces tumor formation (reviewed in Refs. 4–6). Indeed, DFMO has been approved for study as a chemopreventive agent in several types of human cancers (6). In very recent experiments, a transgenic mouse model overexpressing ODC in hair follicle keratinocytes was established. These mice were shown to be much more sensitive to linterm controls to dimethylbenz[a]anthracene-induced carcinogenesis, and did not require treatment with a tumor promoter to develop tumors. The experiments described here focus on a better understanding of the role of ODC in this progression from the normal to the transformed phenotype.

Previous work has shown that ODC activity is induced in response to cellular transformation by oncogenic ras, and levels of ODC in these cells correlate with both the degree of transformation and the number of copies of the ras gene detected. Recently, we have shown that 4E-P2 cells, transformed cells that overexpress translation initiation factor eIF-4E, have high endogenous ODC activity (30 times that in NIH-3T3 cells, from which they are derived; Ref. 11). In addition, p21ras protein is activated in 4E-P2 cells, as shown by an increased proportion of GTP-bound p21ras, although no increase in the synthesis of p21ras was observed (12). Overexpression of GTPase activating protein, a negative regulator of p21ras, reverses transformation of 4E-P2 cells (12). Both DFMO treatment and expression of an ODC dominant negative mutant also abolish the transformed phenotype, suggesting strongly that ODC is important in the neoplastic response (11, 13, 14). Taken together, these results suggest that down-regulation of ODC may nullify the ability of oncogenic ras to transform cells. However, the relationship between Ras activation and ODC induction remains to be determined.

It is well-known that p21ras is an essential component of receptor-mediated signal transduction pathways regulating growth and differentiation, and constitutively active Ras mutants have been implicated in many cancers (reviewed in 15, 16). The Raf/MAPK cascade is thought to play an important role in the transforming activity of oncogenic Ras. This pathway involves the interaction of Ras with the ser/thr protein kinase Raf and activation of a protein kinase cascade resulting in mitogenesis and cell differentiation. However, recent studies have suggested that full malignant transformation by Ras involves not only the Raf/MAPK pathway but also the Raf/Raf family of proteins, which control signaling pathways that regulate actin cytoskeletal organization (18–20). The experiments presented here also examine a Rafα-mediated pathway as a possible intermediary of ODC induction in response to Ras transformation.

Our previous work shows that intracellular ODC activity is reduced by the expression of an inactive ODC protein (K69/A/C360A) in which the key active site residues lysine-69 and cysteine-360 have been converted to alanines (21). ODC is active as a dimer and contains two active sites formed at the interface between monomers. The lysine-69 residue from one monomer interacts with the cysteine-360 from a second monomer to form each active site. K69/A/C360A acts in a dominant negative manner, with mutant and wild type monomers combining to form inactive heterodimers (22). Use of this mutant to lower intracellular ODC activity has advantages over inhibitors such as DFMO, because reduction of enzyme activity does not depend on the continued presence of the inhibitor. Because of both the small residual activity of the K69/A/C360A protein (0.03% of wild type using purified proteins), as well as the presence of wild type homodimers, it is not possible to totally abolish ODC activity, even at high levels of mutant expression (22). This is also an advantage in that it is known that ODC activity is essential for cell viability (4). We have shown that K69/A/C360A expression reverses the transformation...
of 4E-P2 cells (14). In the current experiments, activated mutants of H-Ras, RhoA, and Raf were used to determine which pathways downstream of Ras contribute to the regulation of ODC activity. K69A/C360A was expressed in conjunction with the activated oncogenes to study whether this ODC induction is necessary for transformation by oncogenic ras.

MATERIALS AND METHODS

Plasmids. Plasmids coding for activated mutants of H-Ras and RhoA (pZipras(61L) and pZiprhoA(63L)) were generously provided by Dr. C. J. Der, University of North Carolina, Chapel Hill, North Carolina. The plasmid encoding an activated Raf mutant (LTR-v-raf) was a generous gift of Dr. G. Prendergast, University of Pennsylvania, Philadelphia, Pennsylvania. The K69A/C360A truncated ODC mutant (14) was ligated into the pZeoCMV vector (Invitrogen, Carlsbad, CA) after digestion with XhoI and BsmHI. This vector was designated pZeoCMV/K/C425. All plasmids were purified using Qiagen plasmid purification columns (Qiagen, Inc., Chatsworth, CA).

Cell Culture. Cells were grown in DMEM supplemented with 10% FCS, 4 mM glutamine and 100 μg/ml penicillin and streptomycin. Cell extracts were prepared for enzyme assays as described previously (11).

Stable Transfections. Cells were transfected using the lipofection procedure (Life Technologies, Inc., Gaithersburg, MD) as described previously (14). Between 20–30 individual clones expressing each oncogene (H-Ras, RhoA or v-Raf) were isolated using cloning cylinders (14). Each clone was plated on duplicate 6-cm plates and allowed to grow to confluence, when morphology was assessed as described in the legend to Fig. 1. Cells were then harvested and assayed for ODC activity by measuring the release of 14CO2 from [1-14C] ornithine as described previously (11). Stably transfected cells were maintained in 500 μg/ml geneticin (G418 sulfate; Life Technologies, Inc.) and/or 375 μg/ml zeocin (Invitrogen) throughout the experiment.

Soft Agar Experiments. For plating in soft agar, 5 × 104 cells were resuspended in 2 ml of 0.35% (w/v) agar solution containing DMEM plus 20% FCS, and overlaid onto a 0.5% agar solution in a 35-mm plate. Seven days after plating, 2 ml of DMEM supplemented with 20% FCS was added. Colonies were counted 8–14 days after plating. Colonies are defined as aggregates of more than 10 cells.

Western Blots. Cells expressing LTR-v-raf plus or minus pZeoCMV/K/C425 were maintained in complete medium for 48 h and then were harvested in radioimmunoprecipitation assay buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, aprotinin, and 1 mM sodium orthovanadate), or ODC harvest buffer (50 mM Tris-HCl, pH 7.5, 2.5 mM DTT, 0.1 mM EDTA), and 100 μg of extract was subjected to SDS-PAGE using 12.5% gels. Western blots were performed using chemiluminescent detection systems according to the directions of the manufacturers (Tropix, Bedford, MA) and/or 375 μg/ml zeocin (Invitrogen) throughout the experiment.

RESULTS

Stable Expression of H-Ras and RhoA in NIH-3T3 Cells: Relation to ODC Overexpression. To determine whether high ODC levels are associated with transformation by known oncogenes, NIH-3T3 cells were transfected with plasmids coding for activated mutants of either H-Ras or RhoA. HRas(61L) is an oncogenic Ras mutant that replaces a glutamine at position 61 with leucine. Similarly, RhoA(63L) replaces a glutamine at position 63 with a leucine, and activates Ras-transforming activity. The HRas(61L) mutant is strongly transforming in NIH-3T3 cells, whereas the RhoA(63L) mutant has been reported to form foci at about 10% the rate of HRas(61L) (23).

Cells were stably transfected with either HRas(61L) or RhoA(63L), and approximately 30 clones of each were chosen and allowed to grow for 5 days without changing the medium. Cell morphology of confluent monolayers was first graded on a scale of one to three, with one being normal and three being completely transformed. The ODC activity of each clone was then determined and plotted as a function of the degree of transformation. As seen previously for Ras-transforming mutants (9), there was a good correlation between increasing ODC activity of each clone and change in morphology from normal to spindle-shaped and refractile, some transformed foci; 3 = greater than 90% of cells appear spindle-shaped and refractile, many transformed foci. Each clone was then harvested and assayed for ODC activity, which was plotted as a function of the degree of transformation.
although ODC activities were about 40% lower in the Rho clones and none were completely transformed, as might be expected from information already available suggesting that Rho is only weakly transforming (23, 24). On the other hand, four Ras transfected clones were described as completely transformed, including the two clones in either group with the highest ODC specific activity. There is some degree of overlap between the extent of transformation and ODC activity in both groups. This may reflect different growth rates of the cells that were completely transformed by the criteria described above. In addition, there does not seem to be a direct correlation between the amount of ODC activity and anchorage-independent growth of H-Ras and RhoA Clones. Using stable clones expressing either HRas(61L) or RhoA(63L) and exhibiting a range of ODC activities, experiments examining the formation of colonies in soft agar were performed as described in “Materials and Methods.” Colonies were counted after 8 days of growth, with a colony defined as an aggregate of more than 10 cells. Colony forming efficiency is expressed as the number of colonies counted × 100 divided by the number of cells plated. Parallel plates were grown on a monolayer for 72 h and assayed for ODC activity. Results are the means ± SD of three separate determinations.

Fig. 2. ODC activity correlates with anchorage-independent growth of both H-Ras and RhoA clones. Experiments examining the formation of colonies in soft agar were performed as described in “Materials and Methods.” Colonies were counted after 8 days of growth, with a colony defined as an aggregate of more than 10 cells. Colony forming efficiency is expressed as the number of colonies counted × 100 divided by the number of cells plated. Parallel plates were grown on a monolayer for 72 h and assayed for ODC activity. Results are the means ± SD of three separate determinations.

Stable Expression of K69A/C360A in HRas(61L)-, RhoA(63L)- or v-Raf-transformed Cells. One clone from each group of cells expressing either v-Raf, HRas(61L), or RhoA(63L) was also stably transfected with either the empty pZeoCMV vector or the K69A/C360A-expressing vector pZeoCMVK/C425 and clones expressing each oncogene along with the ODC dominant negative mutant were isolated. All pZeoCMV controls exhibited a transformed morphology when grown in monolayer culture (Fig 3A). Expression of the ODC dominant negative mutant caused the morphology of both HRas(61L) or RhoA(63L)-transformed cells to appear more flat and fibroblastic when observed on a monolayer of cells (Fig 3B). On the other hand, when K69A/C360A was expressed in v-Raf-transformed cells, the cellular morphology was unchanged, and maintained the very refractile appearance of transformed cells (Fig 3B). Ras, Rho and Raf expression levels were verified by Western blots in the pZeoCMVK/C425 clones studied and were not significantly different from levels in pZeoCMV controls (data not shown).

Expression of K69A/C360A almost completely abolished the ODC activity in Rho(63L)-expressing cells compared with empty vector controls, which correlated well with a loss of anchorage-independent growth in these cells (Fig. 4A). Similar results were obtained when K69A/C360A was expressed in HRas(61L)-expressing clones (Fig 4B), although neither ODC levels nor colony formation reached the low levels seen in the Rho + K/C425 cells. In contrast, although expression of the ODC dominant negative mutant was able to reduce
Western blots were performed with an antibody that specifically recognizes phosphorylated p44 and p42 MAPK. All of the groups studied contained measurable levels of phosphorylated p42 MAPK, probably due to autophosphorylation. However, each of the v-Raf transformed clones showed a dramatic induction of phosphorylated p44 MAPK compared with NIH-3T3 control cells (Fig. 6A). In addition, expression of the ODC dominant negative mutant did not reduce the amount of active MAPK in v-Raf transformed cells (Fig. 6A), suggesting that ODC induction in response to Ras transformation

the ODC activity in v-Raf-transformed cells, there was no obvious relationship between ODC activity and the ability of these cells to grow in soft agar (Fig. 4C). These results are not due to loss of expression of the K69A/C360A protein. The expression of the ODC K69A/C360A mutant was confirmed by Western blot analysis of the Raf + K/C425 cells (Fig. 5A), as well as Ras + K/C425 and Rho + K/C425 cells (Fig. 5B and 5C), and levels of the dominant negative ODC were at least as abundant as the endogenous ODC in all cases. The K69A/C360A protein is truncated at 425 amino acids, resulting in a molecular weight of 47 kDa compared with 51 kDa for wild-type ODC.

MAPK Levels in v-Raf-transformed Cells. The amount of active MAPK was determined in several clones of v-Raf-transformed cells with and without the addition of the ODC dominant negative mutant. Western blots were performed with an antibody that specifically recognizes phosphorylated p44 and p42 MAPK. All of the groups studied contained measurable levels of phosphorylated p42 MAPK, probably due to autophosphorylation. However, each of the v-Raf transformed clones showed a dramatic induction of phosphorylated p44 MAPK compared with NIH-3T3 control cells (Fig. 6A). In addition, expression of the ODC dominant negative mutant did not reduce the amount of active MAPK in v-Raf transformed cells (Fig. 6A), suggesting that ODC induction in response to Ras transformation
is either downstream of MAPK or lies on a separate pathway. Similar results were obtained in H-Ras transformed cells (results not shown). When a pan-ERK antibody was used to measure total MAPK levels in the same v-Raf clones, there was no significant difference among any of the groups tested (Fig. 6B). The results shown in Fig. 6A also suggest that, although there is an increase in p44 phospho-MAPK levels in v-Raf-transformed cells, there seems to be a decrease in p42 phospho-MAPK levels. However, this result was not consistent in two subsequent Western blots performed on the same clones (data not shown).

**DISCUSSION**

These results show that the K69A/C360A dominant negative mutant can be used effectively to lower the intracellular ODC activities in oncogene-transformed cells. In addition, K69A/C360A is a valuable tool in determining whether elevated ODC activity is required for transformation by the Ras and Rho families of GTPases. Several groups have established a crucial function for Rho in transformation by oncogenic Ras. It has been shown that coordinate expression of activated RhoA with H-Ras is capable of enhancing both focus formation and growth in soft agar (23). In similar experiments, an activated form of RhoB was shown to synergize in transformation with constitutively activated Raf-CAAX (26), suggesting a two-pathway model for Ras signal transduction. Two H-Ras mutants that are defective in Raf binding and activation have also been shown to induce a transformed morphology similar to that induced by activated Rho mutants (27, 28). Stable transfection of activated Rho mutants into NIH-3T3 cells results in anchorage-independent growth and tumor formation in nude mice (23, 26, 29). Activated Rho mutants are not, however, thought to be activators of the Raf/MAPK pathway (23, 30, 31). In addition, dominant negative mutants of Rho proteins reduce focus formation by oncogenic Ras in NIH-3T3 cells (23, 32) and Rat1 cells (24), and stable coexpression of the dominant negative RhoA(N19) causes about a 50% reversion of Ras transformation in Rat1 fibroblasts (32). Neither RhoA nor Raf dominant negative mutants completely revert the ability of Ras to transform cells, suggesting that multiple signaling events are involved in Ras transformation. Our soft agar experiments agree with this model, because neither v-Raf nor RhoA-transformed cells form colonies as efficiently as cells transformed by H-Ras.

A strong correlation was seen between both HRas(61L) and RhoA(63L) transforming activity and increasing levels of ODC activity. An increase in ODC activity was also observed in v-Raf transformed cells, and this induction followed the degree of transformation as seen for H-Ras and RhoA transformed cells. However, the increase in ODC activity in the highest expressing v-Raf clones was about 10-fold less than that seen in cells transformed by the H-Ras activated mutant. This rise in ODC activity in response to overexpression of v-Raf is not surprising, because it is known that transcription of ODC is activated in response to oncogenes that act through Raf-1, such as v-src (33, 34). However, the results presented here suggest that induction of ODC in response to Ras transformation may involve an alternate pathway. The results observed in RhoA(63L)-expressing cells suggest that this may be a RhoA-mediated pathway. Recent experiments have suggested that several distinct pathways regulated by RhoA are responsible for its effects on cytoskeletal organization, transcription, and cellular transformation. For example, although RhoA has been shown to regulate transcriptional activation by the c-fos serum response factor, activation of serum response factor did not correlate with the ability of RhoA to transform cells (35-37). Possible mediators of RhoA-induced cellular transformation include the protein kinase ROCK-I (37) and nuclear factor-κB, which is transcriptionally activated by RhoA through a mechanism that is independent of Ras and Raf (38). Which of these Rho effectors may be involved in the induction of ODC in RhoA-transformed cells will be the subject of future experiments.

Reduction of intracellular ODC activity by stable transfection of the dominant negative mutant K69A/C360A reverted Ras- and Rho-transformed cells to a more normal phenotype while lowering intracellular ODC activities almost to control levels. In contrast, the phenotype of v-Raf-transformed cells was completely unaffected by coexpression of K69A/C360A, although intracellular ODC activity was also lowered in these clones. Activated MAPK levels were also unaffected in v-Raf-transformed cells expressing K69A/C360A. These results agree with the idea that transformation by Ras involves multiple pathways rather than a linear pathway from Raf to MAPK, because Ras-transformed cells and Raf-transformed cells respond differently to K69A/C360A expression. Furthermore, these results suggest strongly that ODC induction is a necessary step in the process of Ras transformation.

In agreement with the MAPK experiments presented here, it has also been shown that neither p44 nor p42 MAPK is activated in ODC-transformed NIH-3T3 cells (34). However, it has recently been reported that ODC overexpression in MCF-10A breast epithelial cells leads to activation of p42 MAPK and increased anchorage-independent growth (39), and 10T1/2 cells transformed by ODC overexpression show enhanced activity of both p44 and p42 MAPK, as well as increased invasiveness (40). The reason that MAPK is induced in some cell types in response to transformation by ODC and not in others remains to be determined.

It is known that MAPK activation is essential for transformation of NIH-3T3 cells by Ras (41), and our results do not rule out the possibility that ODC induction in response to Ras transformation occurs at a downstream point that requires MAP kinase activation. Höltät and colleagues have suggested based on experiments with tyrosine kinase inhibitors that a novel tyrosine kinase downstream of ODC is responsible for cell transformation induced in response to either H-ras or v-src (33, 42). The current results fit with the idea of a downstream effector of Ras transformation that is part of a pathway including both RhoA and ODC.

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**REFERENCES**


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