Reversible Regulation of the Transformed Phenotype of Ornithine Decarboxylase- and Ras-Overexpressing Cells by Dominant-Negative Mutants of c-Jun

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ABSTRACT

c-Jun is an oncogenic transcription factor involved in the regulation of cell proliferation, apoptosis and transformation. We have previously reported that cell transformations induced by ornithine decarboxylase (ODC) and Ha-ras oncogene, commonly activated in various cancer cells, are associated with constitutively increased phosphorylation of c-Jun on Ser residues 63 and 73. In the present study, we examined the significance of c-Jun phosphorylation and activation on the phenotype of the ODC- and ras-transformants, using specific inhibitors and dominant-negative (DN) mutants to c-Jun NH2-terminal kinase (JNK) and its upstream kinase, SEK1/MKK4 (mitogen-activated protein kinase kinase 4), and to c-Jun. The transformed morphology of both the ODC- and ras-expressing cells was reversed partially by JNK inhibitors and DN JNK1, more effectively by DN SEK1/MKK4 and phosphorylation-deficient c-Jun mutants (c-JunK63A, c-JunK63A,T48A) and most potently by a trans-activation domain deletion mutant of c-Jun (TAM67). Moreover, tetracycline-inducible TAM67 expression in ODC- and ras-transformed cells showed that the transformed phenotype of the cells is reversibly regulatable. TAM67 also inhibited the tumorigenicity of the cells in nude mice. These inducible cell lines, together with their parental cell lines, provide good models to identify the genes and proteins relevant to cellular transformation.

INTRODUCTION

c-Jun is an oncogenic transcription factor that is present as a major component of the AP-1 (activator protein 1) transcription factor complexes. It has an NH2-terminal transactivation domain, a DNA-binding domain, and a leucine zipper domain in the COOH-terminal domain, through which it can dimerize (1, 2). c-Jun is an immediate early gene transcribed rapidly and transiently after stimulation of normal quiescent cells with different kinds of mitogens and tumor promoters. The expression of c-Jun is constitutively increased in many transformed cell lines (1, 2) and human cancers (3), and overexpression of c-Jun alone can induce the transformation of immortalized rodent fibroblasts (4) and chicken embryo fibroblasts (5, 6).

The regulation of c-Jun occurs both transcriptionally and translationally, and its activity can be regulated through phosphorylation and dimerization with different partners. c-Jun is phosphorylated by specific kinases called c-Jun NH2-terminal kinases (JNKs) and in some cell types also by mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)-mediated mechanisms (7, 8). JNKs (including 10 known isoforms) phosphorylate c-Jun within its NH2-terminal activation domain at Ser 63 and 73 (9), which results in an increase in its transcriptional activity and stability (10–12). JNKs (mainly the ubiquitous isoforms 1 and 2) are known to be potently activated by cell exposure to UV radiation, pro-inflammatory cytokines, and environmental stress (13–15) but may also be activated during apoptosis, differentiation, morphogenesis, and oncogenesis (9). JNK activation is triggered by dual phosphorylation on distinct Thr and Tyr residues by two specific MAPK kinases, MKK4 (SEK1) and MKK7 (9). Finally, c-Jun can form homodimers and/or heterodimers, the latter preferred, with other Jun family members (JunB and JunD), Fos family members (c-Fos, FosB, Fra1, and Fra2), activating transcription factor proteins (ATF-2) and other proteins (e.g., Maf). The different complexes formed can then bind either to a 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE; Refs. 16, 17) or to a cAMP-responsive element (CRE; Ref. 17) in the promoters of genes that regulate cell proliferation and differentiation.

The activation of c-Jun seems to be specifically required for progression through the G1 phase of the cell cycle (18–20). Interestingly, in fibroblasts, c-Jun has recently been shown to control the cell cycle by acting as a direct negative regulator of p53 expression (21). However, c-Jun may also act independently of p53 (22). c-Jun has also been shown to be involved in the control of apoptosis (19, 23–25). Not much is yet known about the molecular mechanisms involved, but there is some indication that the regulation of cell proliferation and apoptosis by c-Jun may be governed by at least two distinct pathways (19, 24). Which pathway is selected may depend on the availability and formation of specific c-Jun dimerization complexes or on the other signaling pathways coincidentally activated. In addition, growing evidence in tissue culture and animal tumor models indicate that c-Jun is a central player in the control of cellular transformation. Indeed, the transforming activity of many known and putative oncoproteins, such as activated receptor Tyr kinases, intracellular Tyr and Ser kinases, ras, S-adenylmethionine decarboxylase (AdoMetDC), and nuclear oncoproteins c-Myc and SV40T, appear to be dependent on c-Jun activation (22, 26–32).

Recent studies have shown that ornithine decarboxylase (ODC), a key regulatory enzyme in the biosynthesis of polyamines (putrescine, spermidine, and spermine) is also a protein that, like c-Jun, is essential for mammalian cell proliferation (33–37) and may have role in cell transformation. Overexpression of ODC alone is sufficient to transform immortalized rodent cell lines (38, 39), and, with the ras oncogene, it is able to transform primary cells (40). ODC is also known to be highly activated in cells transformed by various carcinogens and oncogenes, such as v-src, neu, myc, and ras, as well as in a variety of clinical cancers (34–37, 41–43). Furthermore, inhibition of ODC activity with specific inhibitors or by dominant-negative mutants results in inhibition or reversion of transformation by v-src (39, 44) and ras (45, 46). As to the possible mechanisms involved in ODC-induced transformation, we have previously detected constitutive activation of JNK and constitutively increased phosphorylation of the c-Jun transcription factor on Sers 63 and 73 in these transformants (41, 47). These findings are similar to those obtained with ras, v-sti, v-src, raf-1, and polyomavirus middle-sized tumor antigen (13, 48–54), implying an important role specifically for c-Jun phosphorylation in the regulation of transformation.

In the present study, we examined the significance of c-Jun phosphorylation and activation in ODC- and Ha-ras-induced cell transform-
We studied the effects of dominant-negative mutants of SEK1/MKK4 and JNK1, specific JNK inhibitors, two phosphodefi-
cient mutants of c-Jun and a transactivation domain deletion mutant of c-Jun (TAM67) on the transformed phenotype of the cells. We also generated tetracycline-regulatable TAM67-expressing cell lines from the ODC- and ras-transformants, and show that the state of transforma-
tion and tumorigenicity of these cells can be effectively regulated by the expression of TAM67. These inducible cell lines provide good models to identify the molecular mechanisms relevant to cell trans-
formation.

MATERIALS AND METHODS

Cell Culture. NIH3T3 cells stably transfected with the human ODC cDNA (Odc; Ref. 39), the c-Ha-rasV12 oncogene (pGEJ6.6; E4; Ref. 55) and an ODC-transformant derived from the Odc cell line-induced tumors in nude mice (Odc-n; Ref. 56) have been described previously. The cells were grown in DMEM containing antibiotics (penicillin, streptomycin, and gentamicin) and 5% fetal bovine serum (FBS; Bioceil) at 37 °C in a 5% CO2 atmosphere. The normal NIH 3T3 cells were grown in DMEM with antibiotics and 5% FBS or new born calf serum (Life Technologies, Inc.).

The Odc, Odc-n, E4, and normal NIH 3T3 cells, stably transfected with a tetracycline-inducible expression system of c-Jun deletion mutant TAM67 (see below) were cultured as above or in MEM α (α-MEM) supplemented with gentamicin (50 μg/ml) and 5% FBS, TET System Approved FBS (Invitrogen), or newborn calf serum.

Plasmids and Transfections. Empty pcDNA3-vector (Invitrogen), domi-
nant-negative (DN) JNK1 (FLAG-JNK1[APF]; Ref. 13), DN SEK1 (SEK1[AL]; Ref. 57), DN MKK4 (Flag-MKK4[Ala]; Ref. 58), pMT108 (c-Jun), pMT111 (c-Jun with Ser 63 and Ser 73 mutated to alanines), pMT161 (c-Jun with Ser 63, Ser 73, Thr 91, and Thr 93 mutated to alanines; Refs. 59, 60), and pCMV-TAM67 (Ref. 26, 1 μg) were transfected together with a P̄Babe puro selection marker (Ref. 61: 0.1 μg) into the cells (grown on six-well plates) using the LipofectAMINE Plus method according to the manufacturer’s instructions (Life Technologies, Inc.). The transfections were performed with serum for 3 h. The cells were selected for resistance to puromycin (1.5 μg/ml), which was added 2 days after transfection. Pools of transfectants and multiple individual clones (between 5 and 33) were isolated and selected for further analysis.

Generation of Cell Lines Carrying a Tetracycline-Inducible Expression System of the Transactivation Domain Deletion Mutant of c-Jun (pLRT-

The TAM67 mutant of c-Jun, lacking the transactivation domain, was released from pGEM3-T67 with EcoR1, and the fragment was cloned into pBluescript. Fragments in the sense orientation were released by XhoI/Norf digestion and cloned into XhoI/Norf-digested reverse tetracycline-inducible retroviral vector (62). The pLRT-TAM67 plasmid (1 μg) was transfected into the Odc, Odc-n, E4, and normal NIH 3T3 cells (on six-well plates) using LipofectAMINE Plus. Two days after transfection, selection was started with 5 μg/ml blasticidin (Invitrogen) and continued for 1–2 weeks. The stable transfectants were maintained in 1 μg/ml blasticidin. Several clones (trans-
formed foci) were picked up by cylinder cloning for initial screening of the regulation of TAM67 expression, and the best ones were further subjected to single-cell cloning in 96-wells. The cloned cells were tested for their induc-
ibility of TAM67 expression by adding 1 μg/ml doxycycline (Sigma) 1 day after plating and by analyzing 2–3 days thereafter, the expression of TAM67 by immunoblotting and the morphological changes by microscopy.

Cell Lysates. Cells were harvested by scraping and centrifugation, and were washed 1–2 times with PBS. If not processed immediately, the cell pellets were kept frozen at −70 °C until use. For whole cell extracts, the cells were lysed directly in a hot Laemmli sample buffer without 2-mercaptoethanol [62.5 mm Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.1% bromophenol blue]. The samples were sonicated on ice for 10 s, and the lysates were cleared by centrifugation. The protein concentration was determined using the BCA Protein Assay Reagent Kit (Pierce Chemical, Rockford, IL). Finally, 2-mercaptoethanol was added to a final concentration of 5% and the samples were boiled for 5 min.

Western Blotting. The whole cell lysates (20–50 μg) were resolved by SDS-PAGE (10% acrylamide) and were transferred onto 0.2-μm nitrocellulose membrane (Bio-Rad trans-Blot transfer medium). The membranes were incu-
bated in blocking buffer [25 mM Tris (pH 8.0), 125 mM NaCl, 0.1% Tween, 2% BSA, and 0.1% NaN3] for 4–16 h and then with specific antibodies for 2–4 h at room temperature or overnight at 4 °C. The membranes were washed five times with the TBS-NP/T buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% NP40, and 0.05% Tween] and were incubated 30 min at room temperature with horseradish peroxidase-conjugated swine antirabbit IgGs (DAKO), rabbit anti-
mouse IgGs (DAKO) or goat antimouse IgG/IgM (Chemicon) in the TBS-
NP/T buffer. Phospho-p38 MAPK antibody was bridged with biotinylated antirabbit IgG (Dako) and streptavidin-biotinylated horseradish peroxidase complex. The membranes were washed five or six times with the TBS-NP/T buffer, 15–30 min in high-salt buffer [10 mM Tris (pH 8.0), and 300 mM NaCl], and, finally, three times in TBS (10 mM Tris (pH 8.0) and 150 mM NaCl). The protein bands were visualized using enhanced chemiluminescence (ECL) detection system and exposition to FUJI RX film. Equal loading was assessed by staining the membranes with Ponceau S solution (Sigma) and blotting with actin (see below).

Antibodies. c-Jun and the transactivation domain deletion mutant of c-Jun (TAM67) were detected using c-Jun/AP-1 (Ab-1) polyclonal antibody (Onco-
gene Research Products, Calbiochem) and the HA-tagged c-Jun (pMT108) and the phosphorylation-site mutants of c-Jun (pMT 111, pMT 161) with mouse monoclonal antibody to HA (clone 12CA5; Boehringer Mannheim). DN SEK1/MKK4 (SEK1 [AL], Flag-MKK4 [Ala]), and DN JNK1 (FLAG-JNK1 [APF]) were analyzed with rabbit polyclonal antibody to MKK4/SEK1/ JNK1 (Sigma) and with mouse monoclonal antibody to the FLAG-tag, FLAG M2 (Sigma), respectively. Phosphorylated and total p38 MAPK were detected using polyclonal rabbit antibody to phospho-p38 (Thr180/Tyr182; Cell Signaling Technology, Inc.) and p38 (C-20; Santa Cruz Biotechnology), respectively. As a loading control, actin was probed with mouse monoclonal antibody to actin (Ab-1; Oncogene Research Products).

Analysis of Cell Growth. We studied the effect of TAM67 expression on the growth of the Odc, E4, and normal NIH 3T3 cells carrying the tetracycline-inducible expression system by recording the increase in cell number. The cells (5 × 104) were plated in complete medium (α-MEM or DMEM with 5% FBS or newborn calf serum) on 3-cm-diameter dishes in triplicates in the absence or presence of doxycycline (1 μg/ml). During the next 4 days of culture, the cell numbers were determined every 24 h by Coulter counting.

Use of JNK Inhibitors in Cell Culture. 1-2-Stereoisomer of JNK peptide inhibitor 1 (1-JNK Inhibitor 1) and 1-2-stereoisomer of TAT control peptide (1-2-TAT; Alexis Biochemicals) were used at 0, 1, 5, 15, and 25 μM concentrations. The peptides were added daily to the cultures, and the medium was changed every other day. JNK Inhibitor II (SP600125; Calbiochem), a novel, potent catalytic inhibitor (10 mM stock solution made in DMSO), was used at concentrations of 0, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 μM and 0.1% DMSO as a control. The medium was changed every day. The medium was supplemented with 10% serum and mixed with agar (Noble agar; Difco) to yield a 0.35% agar mixture. The mixtures were then overlaid onto 0.7% bottom agar in 24 well plates. Both agar layers were made with or without doxycycline 1 μg/ml. After polymerization, growth medium with or without doxycycline was added, and the medium was replenished twice a week. The colony formation was followed for 2–3 weeks.

Matrigel Invasion Assay. Twenty-four-well plates (Greiner) were coated with 300 μl of Growth Factor Reduced Matrigel (Becton Dickinson Bio-

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vals. The mice were sacrificed before tumors reached 1 cm³ in size or ulcerated. The tumors were weighed, and one-half of them were frozen in liquid nitrogen for molecular biological analyses and one-half were fixed with formalin and embedded in paraffin for histological analyses.

**ODC Assay.** ODC activity was assayed by measuring the release of [14C]CO₂ from L-(1-[14C]ornithine (55).

**RESULTS**

**Dominant-Negative Mutants of SEK1/MKK4 and JNK1 Reverse, with Different Efficacies, the Transformed Morphology of the ODC- and ras-Overexpressing NIH 3T3 Fibroblasts.** We have previously shown that NIH 3T3 cells, transformed by overexpression of human ODC, display constitutive activation of JNK and phosphorylation of c-Jun at Ser63 and 73 (41). To determine the significance of the JNK activation, we transfected ODC-overexpressing cells with dominant-negative mutants of SEK1/MKK4 and JNK1. Expression of MKK4 [Ala] and DN SEK1/MKK4 (SEK1 [AL]) resulted in reversion of the morphology of ODC-transformed cells (Odc; Fig. 1 A, c and d). Similar results were obtained in cells derived from the ODC-overexpressor-induced tumors in nude mice (Odc-n; Fig. 1 B, b). DN JNK1 expression resulted in less prominent changes, causing partial reversion of the ODC-induced morphology (Fig. 1, A, e and B, c). It is possible that the amount of DN JNK1 expression is not sufficient to attain

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Fig. 1. A, the morphology of normal NIH 3T3 cells (a) and of the ornithine decarboxylase (ODC)-transformed cells (Odc) transfected as follows: (b) with an empty vector [Odc + pcDNA3]; (c) with dominant-negative (DN) SEK1/mitogen-activated protein kinase kinase 4 [Odc + MKK4 (Ala)]; (d) with SEK1 (AL) [Odc + SEK1 (AL)]; (e) with DN c-Jun NH₂-terminal kinase 1 (DNJNK1) (Odc + DNJNK1); (f) with wild-type c-Jun (Odc + pMT108); (g) with c-Jun361,73A (Odc + pMT111); (h) with c-Jun561,73A,91,93A (Odc + pMT161); and (i) with a transactivation domain deletion mutant of c-Jun (Odc + TAM67). B, the morphology of cells derived from the ODC-overexpressing NIH 3T3 cell-induced tumors in nude mice (Odc-n) transfected as follows: (a) with an empty vector (+pcDNA3); (b) with SEK1 (AL) [SEK1 (AL)]; (c) with DN JNK1 (+DNJNK1); and (d) with TAM67 (+TAM67). C, the morphology of the Odc cells after incubation of 3 days as follows: (a) without inhibitor (0 μM); (b) with 5 μM L-stereoisomer of JNK peptide inhibitor 1 (L-JNKI 1; 5 μM); (c) with 15 μM L-JNKI 1 (15 μM); (d) with 15 μM L-TAT peptide as a control (L-TAT 15 μM); (e) without inhibitor (0 μM); (f) with 0.5 μM JNK Inhibitor II (0.5 μM); (g) with 1.0 μM JNK Inhibitor II (1.0 μM); (h) without inhibitor (0 μM); and (i) with 0.1% DMSO (the solvent) as a control (DMSO 0.1%). D, the morphology of NIH 3T3 cells transformed by c-Ha-rasVal12 oncogene (E4) after transfection with the following: (a) with an empty vector (+pcDNA3); (b) with DN SEK1/MKK4 [MKK4 (Ala)]; or (c) with SEK1 (AL) [+SEK1 (AL)]; (d) with DN JNK1 (+DN JNK1); (e) with pMT 108 (+pMT 108); (f) with pMT 111 (+pMT 111); (g) with pMT 161 (+pMT 161); and (h) with TAM67 (+TAM67).

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complete inhibition of JNK1 and JNK2, of which the latter may be of more importance for transformation (63, 64). We were, therefore, interested to study also the effects of the recently introduced cell-permeable inhibitors of JNKs: L-JNK Inhibitor 1, which binds to JNK1 and JNK2 with similar affinity and blocks their interaction with c-Jun (65), and JNK Inhibitor II, which potently blocks ($K_i$, 0.19 μM) the activity of all of the JNKs (66). Addition of increasing concentrations of L-JNK Inhibitor 1 (0.1, 5, 15, and 25 μM) to the Odc cell cultures resulted in slight flattening of the morphology of the cells at concentrations higher than 5 μM, whereas the control peptide L-TAT did not have any appreciable effects at the same concentrations (Fig. 1C). Similarly, JNK Inhibitor II caused a partial reversion of the transformed morphology of Odc cells at concentrations higher than 0.5 μM, whereas bare 0.1% DMSO (the solvent) did not have any effect on the cell morphology (Fig. 1C).

The NH₂-Terminal Phosphorylation Site Mutants and Transactivation Domain Deletion Mutant (TAM67) of c-Jun Reverse the Morphology of the ODC- and ras-Transformed Cells. To investigate the role of c-Jun phosphorylation and activation in the ODC- and ras-induced transformations, we used two phosphodefective c-Jun mutants, c-Jun⁵⁶³,⁷³⁷A (pMT 111) and c-Jun⁵⁶³,⁷³⁷A,T⁹¹,⁹³A (pMT 161) and a transactivation incompetent deletion mutant of c-Jun (TAM67) having most of the transactivation domain (amino acids 3–122) deleted (26). A control transfection with wild-type c-Jun (pMT 108) retained the transformed morphology (Fig. 1, A, f and D,e). The expression of the c-Jun mutants pMT 111 and pMT 161, instead, resulted in reversion of the ODC- (Fig. 1A, g and h) and ras- (Fig. 1D, f and g) induced morphology. Finally, in repeated experiments, the expression of the transcriptionally inactive c-Jun mutant TAM67 caused the most significant reversion of both the ODC- (Fig. 1A, i and B,d) and the ras- (Fig. 1D,h) transformed phenotype.

The expression of DN SEK1/MKK4, DN JNK1, wild-type c-Jun (pMT 108), the phosphorylation defective c-Jun mutants (pMT 111 and pMT 161), and TAM67 in the above experiments was verified by Western blot analyses (Fig. 2A, B, and D–F); it was verified as well that DN SEK1/MKK4 is not affecting p38 MAPK activation in Odc cells (Fig. 2C).
The induction of TAM67 expression reversed the transformed morphology of Odc-pLRT TAM67 (at high levels) because of a counterselection response element in its promoter region (16).

The Transformed Phenotype of the ODC- and ras-Transformed Cells Is Reversibly Regulated by Tetracycline-Inducible Expression System of TAM67. Because we did not obtain stable cell lines expressing TAM67 (at high levels) because of a counterselection (growth inhibition; see below), and to obtain more definitive evidence that expression of TAM67 can reverse the ODC- and ras-induced morphology, we generated ODC- and ras-transformed cell lines in which TAM67 could be conditionally expressed. TAM67 was cloned into a reverse tetracycline-regulatable retroviral expression vector (62) and transfected into the ODC- and ras-transformed cells, and multiple (>25) stable cell clones were isolated. In all of the clones, the expression of TAM67 was tightly regulated by doxycycline addition (with no, or only tiny, leakage under the noninduced conditions) as evidenced by immunoblotting (Fig. 3). As in transient transfection experiments, the induction of TAM67 expression resulted in down-regulation of the endogenous c-Jun expression (Fig. 2, E and F). This is likely caused by a transcriptional repression of the c-Jun gene, which is known to have a variant c-Jun response element in its promoter region (16).

The induction of TAM67 expression resulted in reversion of the transformed morphology of Odc-pLRT TAM67 (A, a and b) and E4-pLRT TAM67 cells (B, a and b). TAM67 expression also inhibited the growth of the ODC-transformants in soft agar (A, c and d) and in Matrigel (A, e and f, and B, c and d). Doxycycline (dox) concentration of doxycycline (0.1–2.0 μg/ml) revealed that the expression level of TAM67 correlated with the degree of reversal of the transformed morphology. After withdrawal of doxycycline, the cells returned to the transformed phenotype in 2–3 days, showing that the cellular transformation process can be reversibly regulated. The inducible expression of TAM67 made it possible to study specifically the effects of TAM67 on the ability of the cells to grow anchorage independently in soft agar and Matrigel. The induction of TAM67 in Odc cells markedly inhibited (>90%) cell growth in soft agar, and the colonies were reduced both in number and size when compared with the noninduced cells (Fig. 4A, c and d). Similarly, the invasive growth potential of the ODC- and ras-transformed cells in Matrigel was highly reduced by TAM67 expression (Fig. 4A, e and f, and B, c and d).
In addition to the morphological reversion, the expression of TAM67 resulted in the inhibition of growth of the ODC- (Fig. 5A) and ras-transformed cells. Interestingly, this was not the case with the normal NIH 3T3 cells, in which inducible expression of TAM67 had no significant effect on cell growth (Fig. 5B). Finally, the activity of ODC was found to remain high in the TAM67-transformed Odc cells, both in the transiently transfected cells and in the cells carrying the inducible pLRT TAM67. After induction of TAM67 expression for 2 days, the ODC activity (which correlates with the amount of enzyme protein) was only minimally affected (24% decreased) relative to the noninduced control (Fig. 6). This small decrease in the ODC activity is likely secondary to the TAM67-induced growth inhibition. These data indicate that the reversal of transformation by TAM67 was not due to its blocking the ODC expression but to the inhibition of AP-1 mediated cellular events.

Expression of TAM67 Inhibits Tumor Formation in Nude Mice. Inoculation of Odc-pLRT TAM67 cells s.c. at both flanks of the mice having the control drinking solution (2% sucrose) resulted, in all cases, in rapidly growing tumors, fibrosarcomas with ulcerative tendency (Table 1, Fig. 7A), whereas the mice receiving doxycycline for TAM67 expression system were grown with (+ dox) or without (− dox) doxycycline for two days. The values (nmol/mg protein/h) are means ± SD of four or five cultures from two experiments. The activity of ODC in the normal NIH 3T3 cells is shown for a reference.

Fig. 6. Ornithine decarboxylase (ODC) activity in the ODC-transformants carrying a tetracycline inducible expression system of TAM67. Odc cells with inducible TAM67 expression system were grown with (+ dox) or without (− dox) doxycycline for two days. The values (nmol/mg protein/h) are means ± SD of four or five cultures from two experiments. The activity of ODC in the normal NIH 3T3 cells is shown for a reference.

Table 1

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<tr>
<th>Nude mice</th>
<th>Weight of tumors (left/right flank), g</th>
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<tr>
<td>− dox</td>
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<tr>
<td>1</td>
<td>0.73/0.83</td>
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<tr>
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<td>4</td>
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<td>5</td>
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<td>Mean±SD</td>
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Similarly to that, we have earlier reported for the ODC-transformed parental cells (56), the uninduced Odc-pLRT TAM67 cells (in mice not having doxycycline) could invade locally to striated muscle and fat tissues (Fig. 7B), whereas the small tumors, arisen from the Odc-pLRT TAM67 cells induced to express TAM67 by doxycycline supplementation, showed reduced invasive capacity (Fig. 7C).

DISCUSSION

In our study, dominant-negative mutants of SEK1/MKK4 were found to reverse the morphology of the ODC-transformed cells, implicating that the JNK pathway is relevant to cellular transformation associated with overexpression of ODC. In support of this notion, transfection of DN JNK1 also resulted in a morphological change, but did not fully revert the transformed phenotype. It is possible that DN JNK1 is ineffective at blocking the functions of both JNK1 and JNK2. It is also conceivable that DN SEK1 not only blocks JNK function but also other kinases, resulting in the significant effect observed. The two different JNK inhibitors (i.e., JNK Inhibitor 1 and JNK Inhibitor II), inhibiting both JNK1 and JNK2 (65, 66), resulted also in partial reversion of the transformed morphology; this supports the latter possibility of JNKs not being the only kinases involved in ODC-induced cell transformation. We also found a minor increase in p38 MAPK activation (phosphorylation) in Odc cells, but DN SEK had no effect on the phosphorylation of p38 MAPK, suggesting that DNSEK may still have other unknown sites of action. Similar findings were made in the ras-transformed E4 cells, i.e., that DN SEK1/MKK4 was more effective than DN JNK1 in reversing the transformed morphology. Previous studies in our laboratory have shown that, unlike the ODC-transformants, the ras-transformed E4 cells do not display constitutive activation of JNK, and that the MAPKs extracellular signal-
Indeed, our studies have revealed that the transfection protocol may transfection systems and different TAM67 expression levels achieved. 

For example, differences in NIH 3T3 cell lines or may result, et al. (68) do not see these effects. These discrepancies may reflect 

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Our results show that the expression of TAM67 was accompanied by down-regulation of endogenous c-Jun, presumably as a result of transcriptional repression through the variant AP-1 site in its promoter (16). However, the TAM67 expression has also been reported not to interfere with endogenous c-Jun expression (67). It is possible that the reduction of c-Jun in our study is due to achieving higher expression levels of TAM67. This may result in significant alteration of the composition of the AP-1 complexes.

Curiously, previous reports on the effects of TAM67 on ras-induced transformation have been conflicting. Whereas Brown et al. (26) and Rapp et al. (30) show that TAM67 affects the transformed morphology (rise of transformed cells on ras cotransfection), Janulis et al. (68) do not see these effects. These discrepancies may reflect differences in NIH 3T3 cell lines or may result, e.g., from different transfection systems and different TAM67 expression levels achieved. Indeed, our studies have revealed that the transfection protocol may greatly affect the results of transient transfection experiments, and that there may be a strong selection against the high TAM67 expressers, complicating the analyses. Clearly, the transient transfection assays should be interpreted with caution.

It is also important to note, that most of the previous studies have dealt with the effects of TAM67 on the induction of transformation and not on the established transformed cell lines studied here. Using a reverse tetracycline-inducible expression system, we could definitively establish that TAM67 can normalize the phenotype of the ODC- and ras-transformed cells. TAM67 not only reversed the transformed morphology but also inhibited the proliferation of the ODC- and ras-transformed cells, whereas the growth of the normal cells was not significantly affected by TAM67. TAM67 also blocked the tumorigenic activity of the cells in nude mice. This raises an intriguing possibility that the inhibition of c-Jun/AP-1 by TAM67 could be effectively exploited in cancer gene therapy without having much adverse effect on the normal cells. The finding that TAM67 superceded the effectiveness of DN MKK4/SEK1, DN JNK, JNK inhibitors and phosphodefective c-Jun mutants in transformation reversal is also a persuasive argument for TAM67 exploitation in future cancer therapeutic approaches. Moreover, our studies show that TAM67 can block the invasive capacity of the ODC- and ras-transformed cells. Finally, as TAM67 expression and the transformation state of the cells can be conditionally regulated, the ODC-pLRT TAM67 and ras-pLRT TAM67 cell lines together with their parental transformed and normal cell counterparts, offer powerful tools to explore and characterize the genes crucial for cellular transformation. As an indication of this, our initial screening of about 9500 genes in these cell lines by cDNA microarray analyses have revealed that there is only a surprisingly small number of potential transformation relevant genes.3

ACKNOWLEDGMENTS

We are grateful to Drs. R. J. Davis (University of Massachusetts Medical School, Worcester, MA), D. Bohmann (University of Rochester Medical Center, Rochester, NY), and J.R. Woodgett (Ontario Cancer Institute, Toronto, Ontario, Canada) for providing plasmids. We also thank Dr. Yulong He (University of Helsinki, Helsinki, Finland) for the advice and help with nude mice injections.

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Cancer Res 2004;64:3772-3779.