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Differential Growth Sensitivity to 4-*cis*-Hydroxy-L-proline of Transformed Rodent Cell Lines

Fortunato Ciardiello, Brunella Sanfilippo, Kazuyoshi Yanagihara, Nancy Kim, Giampaolo Tortora, Robert H. Bassin, William R. Kidwell, and David S. Salomon[1]

Laboratory of Tumor Immunology and Biology, National Cancer Institute, NIH, Bethesda, Maryland 20892

ABSTRACT

The effect of 4-*cis*-hydroxy-L-proline (CHP), a proline analogue, on the anchorage-dependent and -independent growth of several transformed rodent cell lines was studied. Mouse NIH-3T3 fibroblasts transformed by a variety of different oncogenes (Ki-*ras*, *mos*, *src*, *fms*, *fes*, *met*, and *trk*) by a DNA tumor virus (SV40) or by a chemical carcinogen (*N*-methylnitrosourea) were all found to be more sensitive (50% inhibitory dose, 20 to 55 μg/ml) to the dose-dependent inhibitory effects of CHP on growth in monolayer culture than were NIH-3T3 cells (50% inhibitory dose, 120 μg/ml). CHP was generally found to be even more effective in inhibiting the growth of these transformed cells as colonies in soft agar than in monolayer cultures. In addition, rat embryo fibroblasts (CREF) and normal rat kidney fibroblasts (NRK) after transformation with a Ki-*ras* oncogene exhibit a similar increase in their sensitivity to CHP-induced growth inhibition. Treatment of NRK cells with transforming growth factor α (TGF-α) and β (TGF-β), which reversibly induces phenotypic transformation of these cells, increases their sensitivity to CHP to a level comparable with that observed in Ki-*ras*-transformed NRK cells (K-NRK). The growth inhibitory effects of CHP are reversible, since removal of CHP results in a normal resumption of cell growth. CHP uptake occurs primarily through the Na^+- and energy-dependent neutral amino acid transport A system, which is 6- to 7-fold more elevated in K-NRK cells compared with NRK cells. Treatment of NRK cells with TGF-α and/or -β increases the uptake of [^{3}H]methylaminoisobutyric acid on the A system to a level that is similar to that found in K-NRK cells. The functions of the Na^+/K^+ and Na^+/H^+ exchange systems are apparently necessary for the enhanced A system activity, since ouabain and amiloride can inhibit the uptake of [^{3}H]methylaminoisobutyric acid in K-NRK cells and in NRK cells treated with TGF-α and/or -β. The activity of the A system is specifically increased in K-NRK and in TGF-α- and/or -β-treated NRK cells, since the other two major neutral amino acid uptake systems, the ASC and the L systems, and the Ly^+ system for basic amino acid uptake show no apparent changes in their activity in NRK cells after treatment with TGF-α and/or -β or in these cells after transformation with the Ki-*ras* oncogene. These results suggest that the differential growth sensitivity to CHP of transformed rodent cells and of normal fibroblasts treated with TGF-α and/or -β is due in part to an elevated uptake of this amino acid analogue on the neutral amino acid transport A system.

INTRODUCTION

CHP[2] is a proline analogue that can be taken up by cells and incorporated during protein synthesis in place of proline. This event can interfere with the normal production and maturation of proteins that are rich in proline, such as collagen (1). In fact, CHP is known to be an inhibitor of collagen synthesis, because the incorporation of this amino acid analogue into procollagen blocks the normal posttranslational hydroxylation of proline residues that are essential for the stabilization of the triple helical configuration of the mature native molecule. The resulting nonhelical collagen that contains CHP in place of *trans*-hydroxyproline is apparently retained within the cell and is ultimately degraded (2). Collagen production and secretion are essential for the growth and survival of several types of cells *in vivo* and *in vitro* (3, 4). For example, CHP can inhibit the growth of normal mammary epithelial cells and can cause regression of well-differentiated rat mammary adenocarcinomas by inhibiting the production of collagen (5, 6). However, it is not yet clear whether CHP can differentially affect the growth of other normal and nontransformed cells or whether the response of normal cells to CHP can be modulated by serum growth factors.

Proline uptake occurs primarily through the neutral amino acid transport A system. This Na^+- and energy-dependent pathway is one of the most extensively regulated cell membrane transport systems (7, 8). In normal cells, for example, the activity of the A system can be modulated by a variety of agents, including hormones and growth factors (9–11). In contrast, most of these adaptive responses to environmental agents are lost in transformed cells. After transformation, the A system activity is constitutively elevated (12–16).

The present study was undertaken to determine whether CHP can affect the growth of several rodent cell lines transformed by various retroviral oncogenes, by a DNA tumor virus, or by a chemical carcinogen. In addition, the study was designed to assess whether normal cells, such as NRK cells, a normal rat kidney cell line, can be sensitized to the effects of CHP by treatment with TGF-α and -β. These latter growth factors were selected because NRK cells treated with TGF-α and -β are able to grow as colonies in soft agar and assume a transformed morphology in monolayer (17, 18). These two phenotypic properties are characteristic of transformed cells. TGF-α and -β are two growth factors that have been circumstantially implicated as proximal effectors of transformation for several different oncogenes (19, 20).

The results of this study demonstrate that transformed cells are more sensitive to the growth inhibitory effects of CHP than are their normal nontransformed counterparts. This phenomenon may be due in part to an elevated uptake of CHP through the neutral amino acid transport A system after transformation or after treatment of normal cells with TGF-α and/or -β.

MATERIALS AND METHODS

Chemicals. Culture media, antibiotics, trypsin, and FBS were obtained from GIBCO (Grand Island, NY). CHP was from Calbiochem-Behring Corp. (La Jolla, CA). [^{3}H]MeAIB (specific activity, 35 to 50 Ci/mmol), [^{3}H]alanine (specific activity, 40 to 60 Ci/mmol), [^{3}H]leucine (specific activity, 45 to 70 Ci/mmol), and [^{3}H]lysine (specific activity, 75 to 100 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Nonlabeled amino acids were purchased from Sigma (St. Louis, MO). Bovine insulin and human transferrin were obtained from Collaborative Research (Lexington, MA); porcine TGF-β was from R and

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[1] To whom requests for reprints should be addressed, at Laboratory of Tumor Immunology and Biology, Building 10, Room 5B43, National Cancer Institute, NIH, Bethesda, MD 20892.

[2] The abbreviations used are: CHP, 4-*cis*-hydroxy-L-proline; TGF, transforming growth factor; FBS, fetal bovine serum; MeAIB, methylaminoisobutyric acid; NMU, *N*-methylnitrosourea; DMEM, Dulbecco's modified Eagle's medium; IMEM, improved minimal essential medium; ID_{50}, 50% inhibitory dose; EGF, epidermal growth factor.

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D Systems (Minneapolis, MN), and human recombinant TGF-α was generously provided by Dr. Rik Derynck, Department of Molecular Biology, Genentech (San Francisco, CA).

Cell Lines. Mouse NIH-3T3 clone 7 cells were provided by Dr. D. Lowy, National Cancer Institute (Bethesda, MD). *mos*-NIH-3T3 is a clone of NIH-3T3 cells transformed with the Moloney murine sarcoma virus; Ki-*ras*-NIH-3T3 are NIH-3T3 cells transformed with the Kirsten murine sarcoma virus; C11 and F2 are two clones of nontransformed cellular revertants isolated from Kirsten murine sarcoma virus-transformed NIH-3T3 cells (21). *fms*-NIH-3T3 are NIH-3T3 cells transformed with McDonough strain of the feline sarcoma virus and were provided by Dr. C. Sherr, National Cancer Institute. *fes*-NIH-3T3 are NIH-3T3 cells transformed with the ST strain of the feline sarcoma virus and were provided by Dr. J. Evan, National Cancer Institute. *src*-NIH-3T3 are NIH-3T3 cells transfected and transformed with a plasmid carrying the v-*src* oncogene and were provided by Dr. S. Anderson, State University of New York (Stony Brook, NY). *met*-NIH-3T3 are NIH-3T3 cells transfected and transformed with a plasmid containing the human *met* oncogene and were provided by Dr. G. Vande Woude, Frederick Cancer Research Facility, National Cancer Institute (Frederick, MD). *trk*-NIH-3T3 are NIH-3T3 cells transfected and transformed with a plasmid containing the human *trk* oncogene and were provided by Dr. M. Barbacid, Frederick Cancer Research Facility. SV40-NIH-3T3 is a clone of NIH-3T3 cells infected and transformed with the SV40 virus. NMU-NIH-3T3 are NIH-3T3 cells transformed by treatment with the chemical carcinogen NMU. CREF is a normal rat embryo fibroblast cell line that was kindly provided by Dr. P. Fischer, Columbia University (New York, NY). K-CREF are CREF cells transformed with the Kirsten murine sarcoma virus. NRK cells are a normal rat kidney fibroblast cell line that was obtained from Dr. J. DeLarco, National Cancer Institute. K-NRK cells are NRK cells that have been transformed with the Kirsten murine sarcoma virus. NMuMg, a normal mouse mammary epithelial cell line, and NMuMg-Ha-*ras*, a transformed derivative of NMuMg, were obtained by transfection of NMuMg cells with a plasmid containing the human activated c-Ha-*ras* proto-oncogene and were provided by Dr. N. Hynes, Ludwig Institute for Cancer Research (Bern, Switzerland) (22).

All cell lines were grown in DMEM supplemented with 10% FBS containing 4 mM glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid, pH 7.4, streptomycin (100 μg/ml), and penicillin (100 units/ml) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Monolayer Growth. Cells were plated in 12 multiwell cluster dishes (Costar, Cambridge, MA) at a density of 2×10^4 to 5×10^4 cells per well in 2 ml of DMEM containing 10% FBS. Twenty-four h later, they were treated with different concentrations of CHP. After 3 days of treatment, the cells were trypsinized and counted in a Coulter counter (model ZBI).

Soft Agar Growth. Cells (2×10^4 per dish) were seeded in 1 ml of 0.3 Difco Noble agar that was supplemented with DMEM and 10% FBS. This suspension was layered over 1 ml of 0.8% agar medium base layer in 35-mm dishes (Costar) and treated with different concentrations of CHP. After 12 days, the cells were stained with nitro blue tetrazolium, and colonies larger than 50 μm were counted with an Artek model 880 colony counter (Artek Systems Corporation, Farmingdale, NY).

Effects of TGF-α and TGF-β on NRK Cell Growth. The growth response of NRK cells to CHP in the absence or in the presence of TGF-α (2 ng/ml) and/or TGF-β (2 ng/ml) was evaluated in monolayer cultures or in soft agar as described above. For serum-free anchorage-dependent growth, cells were allowed to attach to the wells in DMEM containing 10% FBS. Twenty-four h later, the cells were washed and incubated with IMEM containing 4 mM glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH 7.4, streptomycin (100 μg/ml), penicillin (100 units/ml), bovine insulin (10 μg/ml), and human transferrin (10 μg/ml) in the absence or presence of TGF-α (2 ng/ml) and/or TGF-β (2 ng/ml) and exposed to different concentrations of CHP. After 3 days of treatment, the cells were trypsinized and counted in a Coulter counter.

Amino Acid Transport Measurement. The transport assays were essentially performed as previously described by Boerner and Saier (15) with the following modifications. Cells (4×10^4) were seeded in 6-well cluster dishes (Costar) in 3 ml of DMEM with 10% FBS. After 24 h, the cells were washed with serum-free DMEM and cultured in serum-free IMEM containing human transferrin (10 μg/ml) for 16 h. The cells were then treated for the indicated time periods with or without TGF-α (2 ng/ml) and/or TGF-β (2 ng/ml). Before measuring amino acid uptake, the cells were washed and incubated at 37°C for 30 min in transport buffer (10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl, 135 mM NaCl, and 5 mM glucose). Amino acid uptake was initiated by incubating the monolayer cultures in transport buffer containing 3 to 5 μCi of the radiolabeled amino acid at a concentration of 0.1 mM. After 3 min the uptake of [^{3}H]MeAIB was terminated and, after 1 min, the uptake of [^{3}H]alanine, [^{3}H]leucine, and [^{3}H]lysine. Preliminary experiments have shown that uptake was linear during these periods of time for each of the respective radiolabeled amino acids (data not shown). The cells were then extracted with 1.5 ml of ice-cold 10% trichloroacetic acid, and the trichloroacetic acid-soluble amino acids were counted in 10 ml of Hydrofluor scintillation fluid (National Diagnostics, Somerville, NJ) using a Beckman scintillation counter. Total uptake was corrected for nonspecific uptake by measuring the amount of radiolabeled amino acid taken up in the presence of a 250-fold excess of unlabeled amino acid. The data were normalized on the basis of the cell numbers. For [^{3}H]alanine uptake on the ASC system, the assays were performed at pH 6.0 with transport buffer containing 0.1 mM MeAIB to inhibit alanine transport through the A system (15). For [^{3}H]leucine and [^{3}H]lysine uptake, the assays were performed at pH 7.4 with transport buffer containing 135 mM choline chloride instead of NaCl to ensure the specific uptake through the L and Ly^+ systems, respectively (14, 15).

RESULTS

Effects of CHP on the Growth of Rodent Cell Lines. High concentrations of CHP (greater than 400 μg/ml) have been shown to inhibit amino acid incorporation during protein synthesis (1). However, low concentrations of this amino acid analogue (less than 100 μg/ml) were able to specifically inhibit the synthesis and secretion of proline-rich proteins, such as collagen (1, 2). Therefore, the effects of low doses of CHP (5–150 μg/ml) on the anchorage-dependent and -independent growth of a variety of normal and transformed rodent cell lines were assayed. As shown in Table 1, the anchorage-dependent growth in monolayer cultures of mouse NIH-3T3 cells transformed by several different retroviral oncogenes (Ki-*ras*, *src*, *fes*, *fms*, *mos*) by two activated cellular protooncogenes (*met*, *trk*), a DNA tumor virus (SV40), and a chemical carcinogen (NMU) is 2- to 6-fold more sensitive to the growth inhibitory effects of CHP compared with normal NIH-3T3 cells. For example, to achieve a 50% inhibition of growth of Ki-*ras*-NIH-3T3 cells requires approximately 20 μg/ml of CHP, whereas 120 μg/ml are necessary for eliciting the same degree of response in NIH-3T3 cells. Two nontransformed cellular revertants, C11 and F2, that were derived from viral Ki-*ras*-transformed NIH-3T3 cells are relatively insensitive to the growth inhibition induced by CHP (ID_{50}, 120 and 125 μg/ml, respectively) like the parental NIH-3T3 cells. These two revertants are morphologically flat, exhibit contact inhibition of cell growth, do not grow as colonies in soft agar, and are not tumorigenic *in vivo* (21). As shown in Table 1, the majority of the transformed cells are generally 2- to 5-fold more sensitive to the growth inhibitory effects of CHP in soft agar than in monolayer cultures.

A similar degree of differential growth inhibition induced by CHP was also observed between two normal rat fibroblast cell lines, CREF and NRK (ID_{50}, 100 and 90 μg/ml, respectively), and their counterparts that had been transformed by a viral Ki-*ras* oncogene, K-CREF and K-NRK (ID_{50}, 50 μg/ml). The

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Table 1 *Effects of CHP on anchorage-dependent and -independent growth of rodent cell lines*

Cell line	ID_{50} (μg/ml)[a]	
	Monolayer[b]	Agar[c]
Mouse fibroblasts		
NIH-3T3	120 (±5)	NA[d]
C11 (Ki-*ras* NIH-3T3 cellular revertant)	120 (±4)	NA
F2 (Ki-*ras* NIH-3T3 cellular revertant)	125 (±6)	NA
src-NIH-3T3	100 (±2)	30 (±5)
mos-NIH-3T3	50 (±3)	5 (±2)
trk-NIH-3T3	50 (±2)	55 (±5)
fms-NIH-3T3	40 (±4)	25 (±2)
met-NIH-3T3	30 (±3)	40 (±1)
fes-NIH-3T3	20 (±1)	5 (±2)
Ki-*ras*-NIH-3T3	20 (±4)	10 (±1)
SV40-NIH-3T3	25 (±2)	10 (±1)
NMU-NIH-3T3	20 (±1)	5 (±1)
Mouse epithelial cells		
NMuMg	250	NA
NMuMg-Ha-*ras*	55 (±2)	35 (±1)
Rat fibroblasts		
CREF	100 (±5)	NA
K-CREF	50 (±2)	5 (±2)
NRK	90 (±2)	NA
K-NRK	50 (±1)	15 (±2)

[a] Dose that is able to produce a 50% reduction in growth compared with untreated control cultures. Derived from dose-response experiments in which the cells were treated with different concentrations (5 to 150 μg/ml) of CHP. Values, average (±SD) of quadruplicate determinations.

[b] Cells (5×10^4 per well) were seeded in 12 multiwell cluster dishes. After 24 h, they were treated with different concentrations of CHP and counted after 3 days of exposure.

[c] Cells (2×10^4 per well) were seeded in 35-mm dishes in 0.3% agar containing DMEM and 10% FBS and treated with different concentrations of CHP. After 12 days, the colonies were stained with nitro blue tetrazolium and counted with an Artek 880 colony counter.

[d] NA, not assayable because the cells do not grow as colonies in soft agar.

selective growth inhibition by CHP is not restricted to fibroblasts, since a normal mouse mammary epithelial cell line, NMuMg, is relatively insensitive to growth inhibition induced by CHP (ID_{50}, 250 μg/ml). However, these cells acquire an approximately 5-fold enhanced sensitivity to CHP after transformation with an activated c-Ha-*ras* oncogene (ID_{50}, 50 μg/ml) (Table 1).

Effects of TGF-α and/or TGF-β on CHP Sensitivity of NRK Cells. Several different retroviral oncogenes or activated cellular protooncogenes have been shown to induce the production of growth factors such as the TGFs, which may be involved in mediating the action of these oncogenes (18–20). NRK cells are a nontransformed rat kidney fibroblast cell line that can be phenotypically transformed by treatment with TGF-α and -β (17, 18). In the presence of TGF-α and -β, these cells lose contact inhibition of growth in monolayer cultures and are able to grow as colonies in semisolid medium (17). For these reasons, we chose NRK cells as a model system for studying the effects of TGF-α and -β on modulating the intrinsic growth sensitivity of nontransformed cells to CHP.

Fig. 1 illustrates the morphology of control NRK cells, TGF-α-treated NRK cells (2 ng/ml), and K-NRK cells grown in the absence or presence of CHP (50 μg/ml) for 3 days in medium containing 10% FBS. This concentration of TGF-α, 2 ng/ml, was previously found to be optimal for stimulating the anchorage-dependent growth and for promoting the anchorage-independent growth of NRK cells in soft agar (23). Compared with normal NRK cells (Fig. 1*A*), TGF-α-treated NRK cells (Fig. 1*C*), and *ras*-transformed NRK cells, K-NRK cells (Fig. 1*E*) have a similar morphology in that both populations of cells exhibit a fusiform morphology and tend to pile up on each other at confluency. After treatment with CHP, NRK cells exhibit little or no change in their morphology (Fig. 1*B*), whereas TGF-α-treated NRK cells (Fig. 1*D*) and K-NRK cells (Fig. 1*F*) exhibit a flat morphology. In this experiment, as well as in others conducted in the presence of serum, we found that TGF-α alone was sufficient to produce these morphological changes in NRK cells, presumably because there are sufficient amounts of TGF-β and other growth factors, such as platelet-derived growth factor and insulin-like growth factor I, already present in serum (24).

The growth inhibitory effects of CHP on cells are also dose dependent both in monolayer culture and in semisolid medium (Fig. 2). K-NRK cells are about 2.5-fold more sensitive to CHP than NRK cells in monolayer cultures in medium containing 10% FBS (ID_{50}, 35 and 90 μg/ml, respectively) (Fig. 2*A*). Treatment of NRK cells with TGF-α (2 ng/ml) for 3 days increases the sensitivity of these cells to the growth inhibitory effects of CHP to the same degree as that observed in K-NRK cells, since it shifts the ID_{50} from 90 to 34 μg/ml (Fig. 2*A*). In contrast, TGF-β (2 ng/ml) treatment of NRK cells does not produce any effects by itself on modifying the growth response of these cells in the presence or absence of CHP. However, in monolayer cultures under serum-free conditions, K-NRK cells are approximately 16-fold more sensitive to CHP-induced growth inhibition than are the parental NRK cells (ID_{50}, 6 and 100 μg/ml, respectively) (Fig. 2*B*). Treatment of NRK cells in serum-free medium with TGF-α (2 ng/ml) or with TGF-β (2 ng/ml) slightly increases the sensitivity of NRK cells to CHP (ID_{50}, 50 and 54 μg/ml, respectively) compared with untreated cells within the same experiment. The combined treatment with TGF-α and -β has an additive effect to give the same level of sensitivity to CHP (ID_{50}, 8 μg/ml) as that observed in K-NRK cells (Fig. 2*B*).

NRK cells are not able to grow in soft agar in the absence of TGF-α and TGF-β (17). In the presence of serum, NRK cells treated with TGF-α (2 ng/ml) alone grow as colonies in soft agar with the same efficiency as an equivalent number of K-NRK cells (Fig. 2*C*). Addition of exogenous TGF-β (2 ng/ml) to TGF-α-treated NRK cells does not further increase the number of colonies, since there may be a sufficient amount of TGF-β already present in the serum preparations used in this study to cooperate with TGF-α. K-NRK cells are extremely sensitive to the inhibitory effects of CHP in soft agar growth (ID_{50}, 15 μg/ml). NRK cells treated with TGF-α or with TGF-α and -β exhibit the same degree of sensitivity to CHP (ID_{50}, 20 and 18 μg/ml, respectively) as K-NRK cells (Fig. 2*C*).

To determine whether the relative differences in sensitivity to CHP could be due to variations in the rate of growth between these different populations of cells, K-NRK or TGF-α-treated NRK cells (2 ng/ml) were grown for various periods of time with or without CHP (25 μg/ml) in medium containing 10% FBS (Fig. 3*A*). K-NRK cells have the fastest growth rate. TGF-α-treated NRK cells exhibit a growth rate equivalent to that observed in K-NRK cells and approximately 3-fold higher than that of untreated NRK cells. In the presence of CHP, both K-NRK and TGF-α-treated NRK cells grow more slowly and at rates comparable to that observed with nontransformed NRK cells. The growth inhibitory effects of CHP on K-NRK or TGF-α-treated NRK cells were not evident until 48 h after treatment. There was approximately a 50% growth inhibition at this point and during subsequent growth periods. Under serum-free growth conditions, (Fig. 2*B*), treatment of NRK cells with TGF-α (2 ng/ml) for 3 days enhances by approximately 2-fold their growth rate (75,000 cells/well in control *versus* 151,000 cells/well in TGF-α-treated cells, respectively), whereas treatment with TGF-β (2 ng/ml) for 3 days does not have by itself any

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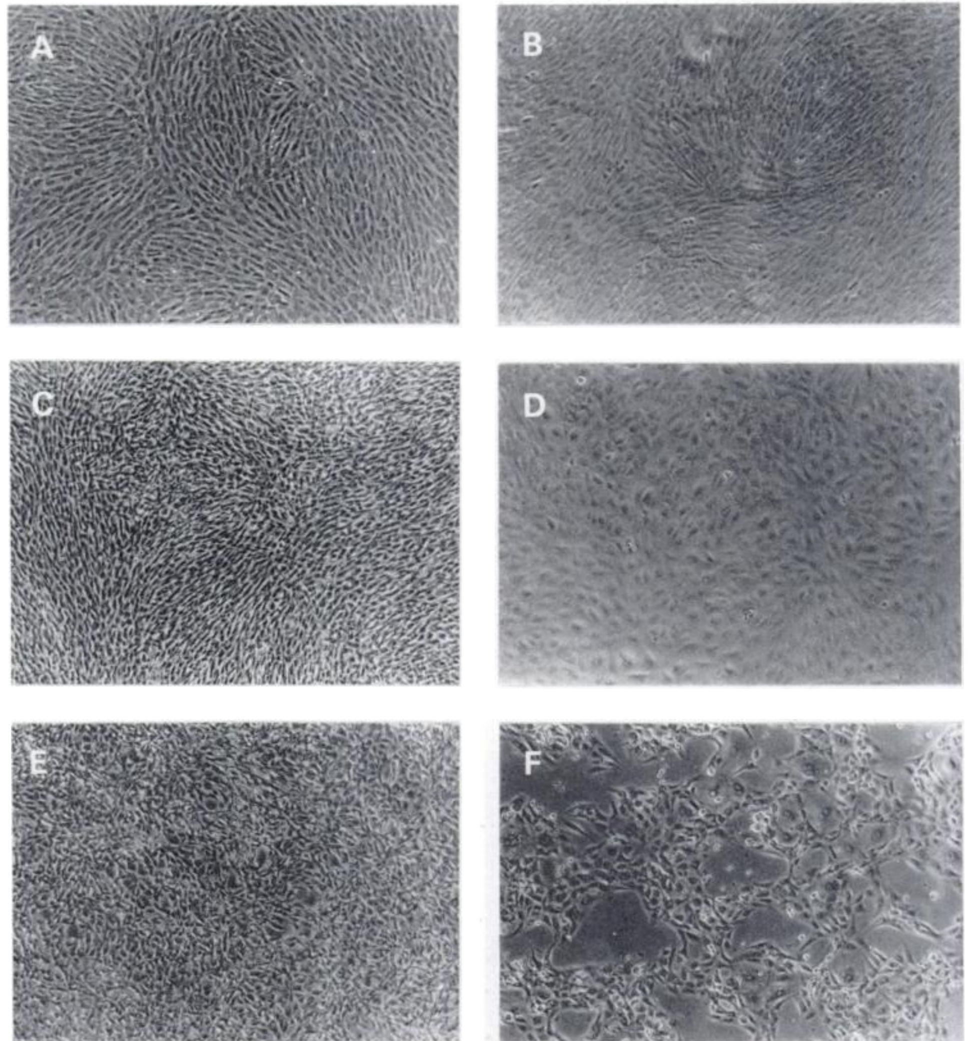

Fig. 1. Effect of CHP on morphology of NRK cells, TGF-α-treated NRK cells, and K-NRK cells. The cells were grown in DMEM containing 10% FBS for 3 days in the absence (*A*, *C*, and *E*) or presence (*B*, *D*, and *F*) of CHP (50 μg/ml). *A*, *B*, NRK cells; *C*, *D*, TGF-α-treated NRK cells (2 ng/ml); *E*, *F*, K-NRK cells.

effect on the growth rate of these cells (82,000 cells/well). Furthermore, the combined treatment of TGF-α and -β for 3 days has a synergistic effect on the growth rate of NRK cells, enhancing it by approximately 10-fold to a level which is comparable to that observed in K-NRK (795,000 cells/well in TGF-α- and -β-treated cells and 775,000 cells/well in K-NRK cells, respectively). However, in the presence of 10% FBS (Fig. 2*A*), TGF-β was ineffective in modifying the growth response of NRK cells to TGF-α.

To determine whether the CHP-induced growth inhibition is cytostatic or cytotoxic, K-NRK cells were grown in the absence or presence of CHP (50 μg/ml) for various periods of time (Fig. 3*B*). CHP was then removed from half of the treated cultures after 3 days. Under these conditions, the CHP-treated cells resume a growth rate equivalent to that of untreated K-NRK cells after 24 h of CHP removal. This indicates that the growth inhibition induced by CHP is reversible and CHP is not cytotoxic but rather cytostatic in our experiments. In addition, trypan blue dye exclusion indicated that CHP was not cytotoxic for either TGF-treated NRK cells or K-NRK cells (data not shown).

Effects of TGF-α and/or -β on Amino Acid Uptake in NRK Cells. CHP is a proline analogue and proline uptake occurs primarily through the neutral amino acid transport A system (7, 8). We therefore studied the characteristics of this amino acid transport system in NRK and K-NRK cells in the presence or absence of TGF-α (2 ng/ml) and/or TGF-β (2 ng/ml) to ascertain whether changes in amino acid uptake through this system could account for the differences in growth sensitivity to CHP of these cells. This may be a rate-limiting step in this response, since the Na^+- and energy-dependent neutral amino acid transport A system can be modulated by various hormones and growth factors and uptake of amino acids through the A system can be increased after cellular transformation (11–16). Fig. 4 demonstrates that TGF-α or -β can rapidly increase the specific uptake of [^{3}H]MeAIB in NRK cells grown in serum-free IMEM containing transferrin (10 μg/ml) by about 5- to 6-fold (20 to 110 pmol/min/10^6 cells and 130 pmol/min/10^6 cells, respectively). MeAIB is a nonmetabolizable analogue of alanine that has been routinely used to measure the activity of the A system at pH 7.4 in the presence of Na^+ (135 mM) (15). Enhanced uptake of [^{3}H]MeAIB is very rapid, reaching a maximum within 1 h after TGF-α treatment and within 3 to 4 h after TGF-β treatment (Fig. 4). [^{3}H]MeAIB remains elevated for at least 16 h after treatment with TGF-α or -β.

To determine whether the activity of the A system is comparable in both NRK and K-NRK cells and whether this activity can be modulated by TGFs, the uptake of [^{3}H]MeAIB was measured in NRK and K-NRK cells that had been treated with TGF-α (2 ng/ml) and/or TGF-β (2 ng/ml) for 4 h. Compared with untreated NRK cells, K-NRK cells exhibited an approximately 7-fold increase in the uptake of [^{3}H]MeAIB (20 and 135 pmol/min/10^6 cells). Treatment of K-NRK cells with TGF-α and/or TGF-β did not affect the uptake of [^{3}H]MeAIB. How-

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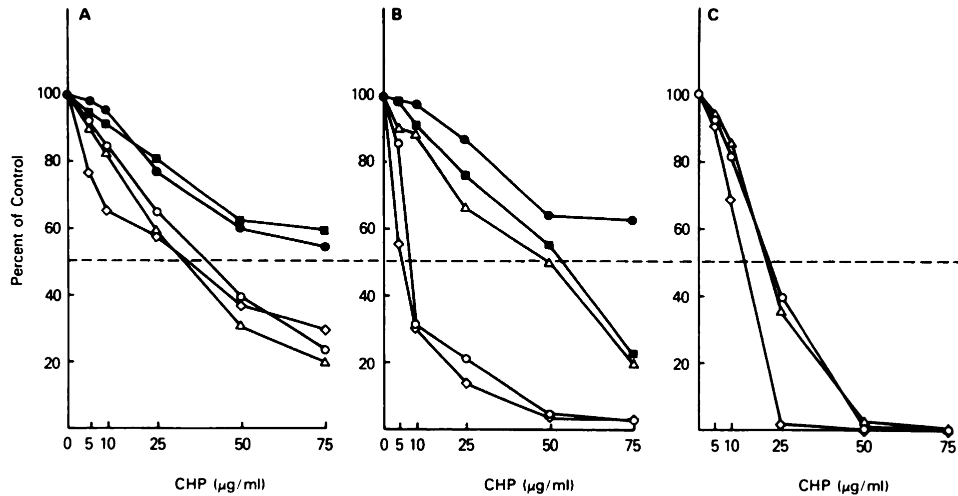

2. Dose-dependent inhibitory effect of CHP on growth of NRK cells, NRK cells treated with TGF-α and/or -β, and K-NRK cells. *A*, monolayer growth in 10% FBS-containing medium. 2 × 10^4 Cells/well were seeded in 12 multiwell cluster dishes. Twenty-four h later, they were treated with different concentrations of CHP and counted after 3 days of exposure. The cell numbers after 3 days of growth for the untreated controls were NRK cells, 144,000 cells/well; NRK cells + TGF-α (2 ng/ml), 421,000 cells/well; NRK cells + TGF-β (2 ng/ml), 156,000 cells/well; NRK cells + TGF-α (2 ng/ml) + TGF-β (2 ng/ml), 442,000 cells/well; and K-NRK cells, 441,000 cells/well. *B*, monolayer growth in serum-free medium. 2 × 10^4 Cells/well were seeded. Twenty-four h later, they were switched to serum-free medium and treated with different concentrations of CHP and counted after 3 days of exposure. The cell numbers for the untreated controls were NRK cells, 75,000 cells/well; NRK cells + TGF-α (2 ng/ml), 151,000 cells/well; NRK cells + TGF-β (2 ng/ml), 82,000 cells/well; NRK cells + TGF-α (2 ng/ml) + TGF-β (2 ng/ml), 795,000 cells/well; and K-NRK cells, 775,000 cells/well. *C*, growth in soft agar. 2 × 10^4 Cells/well were seeded in 0.3% agar containing DMEM and 10% FBS in 35-mm dishes and treated with different concentrations of CHP. The cells were grown for 12 days, stained with nitro blue tetrazolium, and counted with an Artek colony counter. Colonies greater than 50 μm for the untreated controls were NRK cells + TGF-α (2 ng/ml), 1115 colonies/dish; NRK cells + TGF-α (2 ng/ml) + TGF-β (2 ng/ml), 1135 colonies/dish; and K-NRK cells, 1200 colonies/dish. Data are expressed as percentage of cell growth compared with untreated controls. Values, average of 3 different experiments in duplicate. The variation between the individual experiments was less than 10%. ●, NRK cells; △, TGF-α-treated NRK cells (2 ng/ml); ■, TGF-β-treated NRK cells (2 ng/ml); ○, NRK cells treated with TGF-α (2 ng/ml) and TGF-β (2 ng/ml); ◇, K-NRK cells.

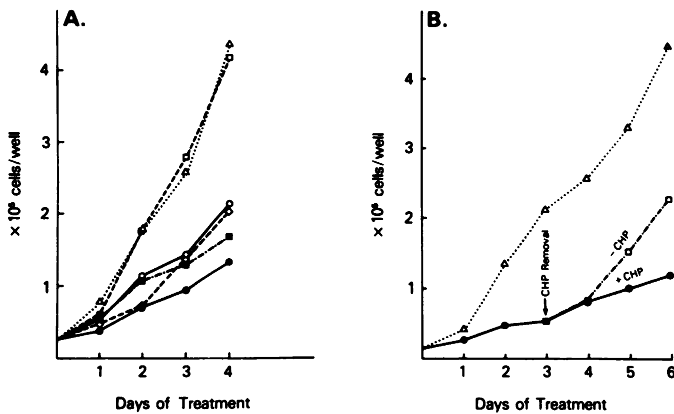

Fig. 3. *A*, effect of CHP on growth curves of NRK cells, TGF-α-treated NRK cells, and K-NRK cells. Cells (2 × 10^4 per well) were seeded in 12 multiwell cluster dishes in DMEM containing 10% FBS. Twenty-four h later, NRK cells in the presence or absence of TGF-α (2 ng/ml) and K-NRK cells were treated with or without CHP (25 μg/ml) and counted every day with a Coulter counter. Results, average of 3 different experiments in duplicate. The variation between the individual experiments was less than 10%. △, K-NRK cells; ◇, CHP-treated K-NRK cells (25 μg/ml); ■, NRK cells; ●, CHP-treated NRK cells (25 μg/ml); □, TGF-α-treated NRK cells (2 ng/ml); ○, NRK cells treated with TGF-α (2 ng/ml) and CHP (25 μg/ml). *B*, reversal of CHP-induced growth inhibition on K-NRK cells. Cells (10^4) were seeded in 12 multiwell cluster dishes in DMEM containing 10% FBS. Twenty-four h later, they were treated with or without CHP (50 μg/ml) and counted every day with a Coulter counter. After 3 days of exposure, CHP was removed from one set of dishes. Results, average of 3 different experiments in duplicate. The variation between the individual experiments was less than 10%. △, K-NRK cells; ●, CHP-treated K-NRK cells (50 μg/ml); □, K-NRK cells rescued from CHP treatment (50 μg/ml) after 3 days.

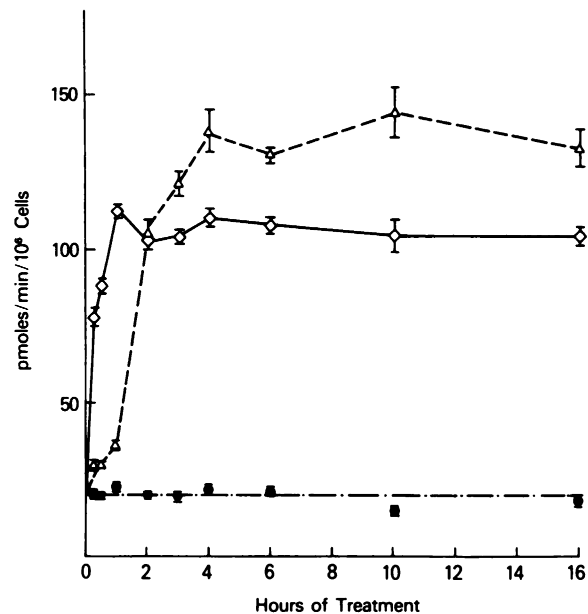

Fig. 4. Kinetics of stimulation of TGF-α or -β on [^{3}H]MeAIB uptake in NRK cells. Cells (4 × 10^4) were seeded in 6 multiwell cluster dishes in DMEM containing 10% FBS. Twenty-four h later, they were washed and cultured in serum-free IMEM containing human transferrin (10 μg/ml) for 16 h. The cells were then treated for the indicated period of time with TGF-α (2 ng/ml) or TGF-β (2 ng/ml), and the [^{3}H]MeAIB uptake was measured as described in "Materials and Methods." Values, average (±SD) of triplicate determinations. ●, NRK cells; ◇, TGF-α-treated NRK cells (2 ng/ml); △, TGF-β-treated NRK cells (2 ng/ml).

ever, treatment of NRK cells with TGF-α or -β increases the uptake of [^{3}H]MeAIB to a level comparable to that observed in K-NRK cells. Furthermore, addition of both TGFs produces an additive effect on the uptake of [^{3}H]MeAIB in NRK cells (205 pmol/min/10^6 cells).

Proline is one of the several neutral amino acids that is taken up on the A system (7, 8). To determine whether the uptake of CHP is occurring primarily through the A system, the ability of CHP, *trans*-hydroxyproline, and proline to inhibit the specific uptake of [^{3}H]MeAIB was measured in NRK cells, NRK cells treated with TGF-α (2 ng/ml) or TGF-β (2 ng/ml), and K-NRK cells. All three amino acids are effective in inhibiting the specific uptake of [^{3}H]MeAIB (data not shown).

The neutral amino acid transport A system is a Na^+- and energy-dependent system (7). It has been suggested that these requirements are controlled in part by the Na^+/H^+ antiporter

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and Na^+/K^+ ATPase (25). Amiloride is a specific inhibitor of Na^+/H^+ exchanger, while ouabain is a specific inhibitor of Na^+/K^+ ATPase (26, 27). Each of these two drugs was therefore tested for its ability to modify the uptake of [^{3}H]MeAIB in NRK cells, NRK cells treated with TGF-α (2 ng/ml) or TGF-β (2 ng/ml), and K-NRK cells. Cells were treated with or without amiloride (1 mM) or ouabain (1 mM) for 4 h (Fig. 5). Untreated NRK cells exhibited an intrinsic low uptake of [^{3}H]MeAIB that was not substantially modified by amiloride and/or ouabain treatment. However, in NRK cells treated with TGF-α or -β or in K-NRK cells, amiloride or ouabain each produces a 45–55% inhibition of [^{3}H]MeAIB uptake. Furthermore, addition of both amiloride and ouabain to TGF-α- or -β-treated NRK cells or to K-NRK cells completely abrogates the effects of these two growth factors on stimulating the [^{3}H]MeAIB uptake in NRK cells and totally blocks the elevated basal uptake of [^{3}H]MeAIB observed in K-NRK cells (Fig. 5).

To determine whether there are any alterations in the regulation of the three other major amino acid transport systems after transformation or TGF treatment of NRK cells, we measured the uptake of [^{3}H]alanine on the ASC system, [^{3}H]leucine on the L system, and [^{3}H]lysine on the Ly^+ system. As shown in Fig. 6, no differences in the uptake of [^{3}H]alanine, [^{3}H]-leucine, or [^{3}H]lysine could be observed between K-NRK cells and NRK cells treated with either TGF-α (2 ng/ml) and/or TGF-β (2 ng/ml) for 4 h.

DISCUSSION

The present study demonstrates that the proline analogue CHP is a potent inhibitor of the growth of transformed rodent fibroblast and epithelial cells. Treatment with low doses of CHP inhibits in a dose-dependent fashion the anchorage-dependent and -independent growth of these transformed cells, whereas CHP is less effective in inhibiting the growth of non-transformed rodent fibroblast and epithelial cells. CHP is more effective in arresting the anchorage-independent growth than the anchorage-dependent growth of these cells. The mechanism(s) by which CHP selectively blocks the growth of these transformed cell lines may be related to its ability to selectively inhibit collagen production and secretion at low concentrations that were used in this study (5, 6). However, several points suggest that this may not be the case. First, normal rodent fibroblast cell lines generally synthesize and secrete low levels of collagen *in vitro* (28). Second, the levels of collagen production and of other extracellular matrix proteins are substantially reduced in fibroblasts after transformation (28–30). Finally, the sensitivity of Ki-*ras*-transformed 3T3 cells to the growth-inhibiting effects induced by CHP is not affected by culturing the cells on dishes coated with type I or IV collagen, suggesting that the cells cannot be rescued from the cytostatic effects of CHP by providing them with an appropriate exogenous collagen matrix (see Ref. 3).[3] It therefore seems unlikely that the growth inhibitory effect induced by CHP is primarily due to changes in levels of collagen production. It is conceivable that CHP is inhibiting the synthesis of other structural or regulatory proteins that contain proline or hydroxyproline. Nevertheless, we are presently conducting experiments to address these issues, since TGF-α and -β have been reported to stimulate collagen production in NRK cells (31, 32), which may partially contribute to their ability to enhance the sensitivity of these cells to the cytostatic effects of CHP.

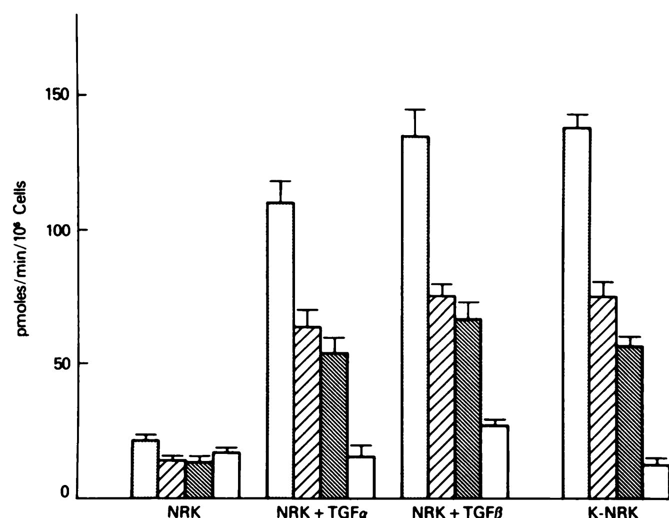

Fig. 5. Inhibition of [^{3}H]MeAIB uptake with ouabain and/or amiloride treatment in NRK cells, NRK cells treated with TGF-α or -β, and K-NRK cells. Cells (4×10^4 per well) were seeded in 6 multiwell cluster dishes. Twenty-four h later, they were washed and cultured in serum-free IMEM containing human transferrin (10 μg/ml) for 16 h. NRK cells, TGF-α-treated NRK cells (2 ng/ml), TGF-β-treated NRK cells (2 ng/ml), and K-NRK cells were then incubated for 4 h in the absence (□) or presence of ouabain (1 mM) (▨) or amiloride (1 mM) (▩) or of both ouabain and amiloride (□). The [^{3}H]MeAIB uptake was measured as described in "Materials and Methods." Values, average (±SD) of three determinations.

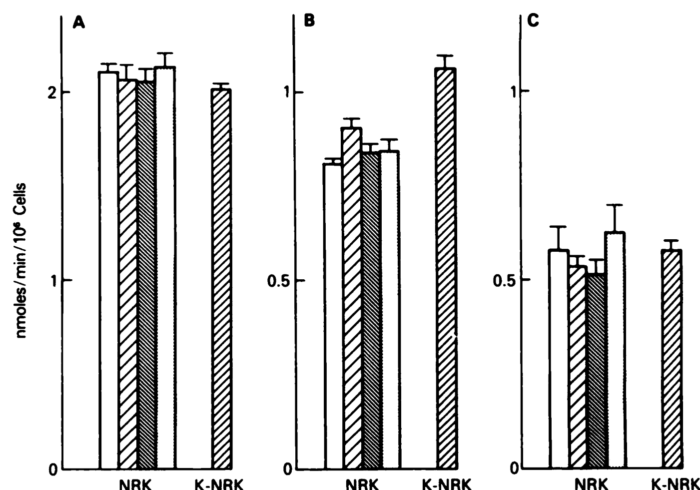

Fig. 6. Amino acid uptakes through the ASC, L, and Ly^+ systems in NRK cells, NRK cells treated with TGF-α and/or -β, and K-NRK cells. *A*, [^{3}H]alanine uptake through the ASC system; *B*, [^{3}H]leucine uptake through the L system; *C*, [^{3}H]lysine uptake through the Ly^+ system. The cells were grown as described in Fig. 4, and amino acid uptake was measured as reported in "Materials and Methods." NRK cells were treated for 4 h with growth factors before measuring the specific uptake of amino acids in the absence (□) or presence of TGF-α (2 ng/ml) (▨) or TGF-β (2 ng/ml) (▩) or of both TGF-α and β (□). Values, average (±SD) of three determinations.

TGF-α and -β have been circumstantially implicated in the autocrine growth of several types of virally, chemically, and spontaneously transformed fibroblasts and epithelial cells (33–38). In normal mesenchymal and in some epithelial cells, TGFs reversibly induce several properties associated with the transformed phenotype, such as a loss of contact inhibition of cell growth, a decreased requirement for serum for anchorage-dependent growth, and the ability to grow as colonies in soft agar. In fact, insertion of an expression vector containing the gene for human TGF-α into normal rat 1 fibroblasts results in the anchorage-independent growth of these cells and in the induction of tumor formation in nude mice (39). Moreover, anti-human TGF-α antibodies are able to inhibit the anchorage-independent growth of TGF-α-expressing clones of rat 1 fibro-

[3] D. S. Salomon and F. Ciardiello, unpublished results.

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blasts, suggesting that TGF-α secretion and action are through an autocrine mechanism. Our results demonstrate that treatment of NRK cells with TGF-α and/or -β also increases their sensitivity to the growth inhibitory effects of CHP to the same relative degree as that observed in Ki-*ras*-transformed NRK cells. The enhanced sensitivity to the growth inhibition induced by CHP of the transformed rodent cells may be related in part to their ability to endogenously synthesize and secrete high levels of biologically active TGF-α and -β. This may be the case, since both fibroblast and epithelial cells transformed by Ki-*ras*, Ha-*ras*, *mos*, *fms*, and *fes* have been demonstrated to secrete elevated levels of TGF-α and -β (19, 33, 35–37).

TGF-α is a potent mitogen for the anchorage-dependent and -independent growth of NRK cells in the presence of serum (23, 40). In fact, TGF-α-treated NRK cells exhibit a similar rate of growth in monolayer cultures and grow as colonies in soft agar to the same degree as that observed in K-NRK cells. In our studies, treatment of NRK cells in serum-containing medium with TGF-β alone or in the presence of TGF-α did not appreciably affect their anchorage-dependent or -independent growth, presumably because there were sufficient amounts of TGF-β in the FBS preparations that we have used in this study (24). Furthermore, in the presence of serum, addition of TGF-β alone did not modify the response of NRK cells to the growth inhibitory effects of CHP in monolayer cultures. In contrast, TGF-α alone, in the presence of serum, was sufficient to enhance the efficiency of CHP to inhibit the growth of NRK cells to the same degree as that observed in K-NRK cells, both under anchorage-dependent and -independent growth conditions. Under serum-free conditions, TGF-α was less effective in stimulating the growth of NRK cells than in the presence of serum. However, in this case, the addition of TGF-β, which by itself did not affect the anchorage-dependent growth of NRK cells, synergistically enhanced the growth-promoting effects of TGF-α. In fact, under the serum-free assay conditions of these studies, both TGF-α and -β were required for achieving maximal growth stimulation of NRK cells. These results are in accord with the observations of Assoian, who has shown that TGF-β may function synergistically with EGF (and presumably TGF-α) to stimulate NRK cell growth by enhancing the level of expression of EGF receptors (41). TGF-α or -β was able to increase the sensitivity of NRK cells to the growth inhibitory effects of CHP to the same degree in serum-free medium. Addition of both growth factors to NRK cells sensitized these cells to CHP-induced growth inhibition to the same extent as that observed in K-NRK cells. Collectively, these results suggest that the sensitivity of growth factor-treated NRK cells or of transformed K-NRK cells to CHP-induced growth inhibition may be related to or may be dependent on the intrinsic growth rate of these cells under the various assay conditions. The only exception to this possibility is the effect of TGF-β on enhancing the response of NRK cells to the growth inhibitory effects of CHP under serum-free culture conditions, since TGF-β alone has no appreciable effect on cell growth but is capable of enhancing the uptake of [^{3}H]MeAIB and presumably CHP.

Among the biochemical features that are frequently observed in fibroblasts treated with growth factors or in transformed cells are an enhanced rate of aerobic glycolysis and an increased uptake of ions, glucose, and amino acids (11, 12, 14, 15, 42). One major amino acid uptake system that is highly susceptible to modulation by growth factors and constitutively elevated in transformed cells is the neutral amino acid transport A system (11–16). The A system is a Na^+- and energy-dependent uptake system for proline, methionine, alanine, serine, and glycine (7, 8). Since CHP is a proline analogue, [^{3}H]MeAIB uptake was analyzed to ascertain whether this may be one of the limiting steps that might account for the differential sensitivity to the growth inhibition induced by CHP between normal and transformed cells or normal cells treated with TGF-α and/or -β under serum-free conditions. The present study demonstrates that the [^{3}H]MeAIB uptake is 6- to 7-fold higher in K-NRK cells than in normal NRK cells. However, treatment of NRK cells with TGF-α or -β produced a rapid and protracted increase in [^{3}H]MeAIB uptake. The response to either growth factor could be observed within 30 min of treatment and within 1 to 3 h reached a level that was 5- to 6-fold higher than in untreated NRK cells and that was comparable to the basal level of [^{3}H]-MeAIB uptake observed in K-NRK cells. In contrast, K-NRK cells were refractory to the stimulation of [^{3}H]MeAIB uptake induced by TGF-α and/or -β. This may be due to the constitutively enhanced [^{3}H]MeAIB uptake observed in K-NRK cells that may be regulated in part by the endogenous production of TGFs in these cells (33, 35). These data are in reasonable agreement with a previous report of Boerner *et al.* demonstrating a 2- to 3-fold enhancement in MeAIB uptake in confluent cultures of NRK cells after treatment with EGF or TGF-β in the presence of serum (11). Our results further extend these observations, since in the present study sparse cultures of NRK and K-NRK cells were treated with TGF-α and/or -β under serum-free conditions to avoid any interference by other serum-derived growth factors or hormones that could potentially modulate the activity of the A system (7). In addition, we were unable to discern a stimulatory effect of TGF-β on the specific uptake of MeAIB in subconfluent NRK cells grown in the presence of serum (data not shown) or on the CHP-induced growth inhibition of NRK cells under these culture conditions. This may relate to the ability of TGF-β to stimulate NRK cell growth depending upon whether the cultures are subconfluent or confluent (31, 41).

CHP effectively competed with [^{3}H]MeAIB for uptake through the A system. This demonstrates that the bulk of CHP is probably being taken up by the neutral amino acid transport A system in these cells. Analysis of [^{3}H]alanine uptake on the ASC system, [^{3}H]leucine uptake on the L system, and [^{3}H]lysine uptake on the Ly^+ system shows that there is no appreciable difference in the total activity of these other three major amino acid uptake systems between normal NRK cells and transformed K-NRK cells. In addition, treatment of NRK cells with TGF-α and/or -β did not significantly enhance the specific uptake of [^{3}H]alanine, [^{3}H]leucine, and [^{3}H]lysine, suggesting that the ASC, L, and Ly^+ systems are constitutively enhanced in normal and transformed fibroblasts and are relatively insensitive to treatment with growth factors such as TGF-α and/or -β (15). These results agree with those of Boerner *et al.*, who have shown that EGF or TGF-β fails to significantly stimulate amino acid uptake through these other three amino acid uptake systems in confluent cultures of NRK cells (11). Furthermore, MeAIB uptake was found to be elevated in rat 1 fibroblasts transformed with an activated c-Ha-*ras* oncogene when compared with nontransformed rat 1 cells and was refractory to stimulation by TGF-β (42).

There is a substantial amount of evidence suggesting that growth factors, after interaction with their respective receptors, can rapidly activate the exchange of Na^+ for H^+ via the Na^+/H^+ antiporter, which results in a transient alkalinization of the cell (43–46). This response may be important for subsequent DNA synthesis induced by growth factors (45, 46). The presence of a viral or activated *ras* oncogene product may control

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either directly or indirectly the activity of Na^+/H^+ antiporter (47). Furthermore, the Na^+/H^+ antiporter (47) is constitutively activated in P19 embryonal carcinoma cells and is insensitive to modulation by growth factors, such as EGF (48). However, differentiation of these cells into nontransformed endodermal cells results in the acquisition of an EGF-stimulated Na^+/H^+ antiporter activity. The enhanced activity of the Na^+/H^+ antiporter system after growth factor treatment or after transformation may lead to an increased activity of the Na^+/K^+ ATPase (25, 42). Our results demonstrate that addition of either amiloride or ouabain can inhibit the constitutive uptake of [^{3}H] MeAIB observed in K-NRK cells. Likewise, both of these inhibitors can attenuate the TGF-α- or -β-induced uptake of [^{3}H]MeAIB in serum-free grown NRK cells. Addition of both amiloride and ouabain to K-NRK cells or to TGF-α- or -β-treated NRK cells reduces the uptake of [^{3}H]MeAIB to a level comparable to that observed in nontransformed unstimulated NRK cells. These data circumstantially suggest that the Na^+/H^+ exchanger and the Na^+/K^+ ATPase are in some manner involved in the control of the activity of the amino acid transport A system in NRK cells after transformation or treatment with growth factors such as TGF-α or -β.

In summary, this study demonstrates that a number of transformed rodent cells or growth factor-treated normal cells are more sensitive to the growth inhibition induced by CHP. This differential sensitivity may be due in part to a more elevated uptake of CHP through the neutral amino acid transport A system after transformation or treatment of normal cells with TGF-α and/or -β. In addition, the data suggest that the sensitivity of various tumor cells to other drugs that are taken up on the A amino acid transport system may possibly be modulated by locally derived growth factors.

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Differential Growth Sensitivity to 4-*cis*-Hydroxy-l-proline of Transformed Rodent Cell Lines

Fortunato Ciardiello, Brunella Sanfilippo, Kazuyoshi Yanagihara, et al.

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