Post-translational incorporation of the antiproliferative agent azatyrosine into the C-terminus of α -tubulin

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Detyrosination/tyrosination of tubulin is a post-translational modification that occurs at the C-terminus of the α -subunit, giving rise to microtubules rich in either tyrosinated or detyrosinated tubulin which coexist in the cell. We hereby report that the tyrosine analogue, azatyrosine, can be incorporated into the C-terminus of α -tubulin instead of tyrosine. Azatyrosine is structurally identical to tyrosine except that a nitrogen atom replaces carbon-2 of the phenolic group. Azatyrosine competitively excluded incorporation of [¹⁴C]tyrosine into tubulin of soluble brain extract. A newly developed rabbit antibody specific to C-terminal azatyrosine was used to study incorporation of azatyrosine in cultured cells. When added to the culture medium (Ham's F12K), azatyrosine was incorporated into tubulin of glioma-derived C6 cells. This incorporation was reversible, i.e. after withdrawal of azatyrosine, tubulin lost azatyrosine and reincorporated tyrosine. Azatyrosinated tubulin self-assembled into microtubules to a

similar degree as total tubulin both *in vitro* and *in vivo*. Studies by other groups have shown that treatment of certain types of cultured cancer cells with azatyrosine leads to reversion of phenotype to normal, and that administration of azatyrosine into animals harbouring human proto-oncogenic c-Ha-ras prevents tumour formation. These interesting observations led us to study this phenomenon in relation to tubulin status. Under conditions in which tubulin was mostly azatyrosinated, C6 cells remained viable but did not proliferate. After 7–10 days under these conditions, morphology changed from a fused, elongated shape to a rounded soma with thin processes. Incorporation of azatyrosine into the C-terminus of α -tubulin is proposed as one possible cause of reversion of the malignant phenotype.

Key words: azatyrosine, microtubule, revertant cell, tubulin, tyrosination state.

INTRODUCTION

Microtubules are dynamic assemblies of tubulin present in all eukaryotic cells, and are involved in spindle formation, chromosome segregation, cell motility, organelle transport, morphology, secretion and signal transduction. Tubulin, both assembled and non-assembled, undergoes several post-translational modifications, among which tyrosination/detyrosination at the C-terminal position of the α -subunit has been extensively studied [1–6]. In assembled tubulin, the C-terminal tyrosine of the α -subunit can be removed by tubulin carboxypeptidase, exposing a glutamic acid residue (forming Glu-tubulin) [7–9]. Conversely, non-assembled Glu-tubulin serves as a substrate for tubulin tyrosine ligase which re-incorporates tyrosine into the C-terminal position. The ligase has been purified, cloned and sequenced [10–13]; however, the carboxypeptidase has not been successfully purified and a specific antibody is not yet available. Although the physiological role of the tyrosination/detyrosination cycle remains unclear, possible roles of the α -tubulin C-terminus have been proposed in regulation of the cell cycle [14] and in differentiation of muscle cells [15]. One of our goals is to detect alterations in normal cell functioning after substitution of the C-terminal tyrosine by various tyrosine analogues. The nature of such alterations could help clarify the role of the tyrosination/detyrosination cycle. *In vitro* incorporation of tyrosine analogues (e.g. phenylalanine, L-dopa, 3-iodo-tyrosine, 3-fluoro-tyrosine, 3-nitro-tyrosine) into the α -tubulin C-terminus has been described [16–20], but in most cases effects on living cells were not studied. We reported recently that 3-nitrotyrosine can be incorporated into tubulin of several cell lines

without a detrimental effect on cell division [21]. On the other hand, nitrotyrosine was shown to affect the tyrosination state of microtubules during myogenic differentiation [15].

The present study was focused on azatyrosine, a compound in which the carbon at position 2 of the phenolic group of tyrosine is replaced by nitrogen [L- β -(5-hydroxy-2-pyridyl)-alanine]. Azatyrosine was first described as an antibiotic produced by *Streptomyces chibaensis* [22], but is more notable for its ability to convert transformed cells to normal phenotype [23]. Growth of NIH-3T3 cells transformed with c-Ha-ras, c-raf, or c-erbB-2 is inhibited by azatyrosine, and their phenotype reverts to normal. Human pancreatic and colonic carcinoma cells (PSN-1 and HCT116, respectively), which have a point mutation in c-K-ras, are also converted to normal phenotype by treatment with azatyrosine [24]. On the other hand, cells transformed by *hst*, *ret* or *src* are not reverted by azatyrosine [23]. There is some evidence that azatyrosine inhibits the main pathway of Ras-mediated signal transduction; that is, it inhibits oncogenic Ras-induced outgrowth of neurites in PC12 cells [25], and Ras-induced maturation of *Xenopus laevis* oocytes [26]. Labelled [³H]azatyrosine was incorporated into several proteins in living cells [27], and such incorporation was competitively blocked by unlabelled tyrosine. High concentrations of tyrosine suppressed the ability of azatyrosine to revert c-ras-transformed cells. These results are consistent with the concept that azatyrosine is incorporated into protein via tyrosyl-tRNA synthetase during *de novo* protein synthesis. However, the mechanism of action of azatyrosine, and its major targets, remain unknown. We hereby demonstrate that azatyrosine can be post-translationally incorporated into the C-terminal

Abbreviations used: Tyr-tubulin, tubulin whose α -subunit has a C-terminal tyrosine residue; Glu-tubulin, tubulin with α -subunit lacking the C-terminal tyrosine residue, exposing the C-terminal glutamic acid residue; Azatyr-tubulin, tubulin whose α -subunit has a C-terminal azatyrosine residue instead of tyrosine.

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position of α -tubulin. Such tubulin modification is a viable alternative mechanism to explain the reverting effect of azatyrosine on transformed cells.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, chemicals and culture media were purchased from Sigma (St. Louis, MO, U.S.A.). L-[14 C]Tyrosine (specific radioactivity, 450 μ Ci/ μ mol) was from New England Nuclear and L-[35 S]methionine (specific radioactivity, 1000 Ci/mmol) was from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Fluorsave was from Calbiochem (La Jolla, CA, U.S.A.). 2-Azatyrosine was a gift from Dr S. Nishimura (Banyu Tsukuba Research Institute, Ibaraki, Japan), Dr A. G. Myers (Harvard University, Cambridge, MA, U.S.A.) and Dr R. S. Phillips (University of Georgia, Athens, GA, U.S.A.).

Soluble rat brain preparation

Brains from 15–30-day-old Wistar rats were homogenized in 1 vol. of MEM buffer (100 mM Mes adjusted with NaOH to pH 6.7, containing 1 mM EGTA and 1 mM MgCl_2). The homogenate was centrifuged at 100 000 g for 1 h and the supernatant solution was passed through a column of Sephadex G-25-80 equilibrated with MEM buffer to eliminate low-molecular-mass compounds. Tubulin concentration in this preparation was approx. 2 mg/ml.

In vitro incorporation of [14 C]tyrosine or unlabelled azatyrosine into tubulin

Unless otherwise specified, the incubation medium contained, per ml, 0.9 ml of soluble brain extract, 2.5 μ mol of ATP, 12.5 μ mol of MgCl_2 , 30 μ mol of KCl, 100 μ mol of Mes buffer, pH 6.7, and 6.7 nmol (3 μ Ci) of [14 C]tyrosine or azatyrosine in the amounts stated for each experiment. Incubation was at 37 °C. At the stated times, aliquots were inactivated by addition of 2 ml of 5% trichloroacetic acid and heated at 90 °C for 15 min. Radioactivity bound to protein was measured in terms of radioactive trichloroacetic acid-insoluble material as described previously [28]. To determine incorporation of unlabelled tyrosine or azatyrosine into tubulin, samples were subjected to immunoblot using anti-Tyr or anti-Azaty antibodies as described below.

Antibodies

Rabbit polyclonal antibody specific to Glu-tubulin (anti-Glu) was prepared as described previously [6]. Polyclonal antibody specific to azatyrosine (anti-Azaty) was raised in rabbits by the technique described for antibodies specific to C-terminal 3-nitrotyrosine [21]. In brief, azatyrosine was bound through its amino group to keyhole limpet haemocyanin, using glutaraldehyde as a cross-linker. The resulting protein (500 μ g) was mixed with complete Freund's adjuvant (1:1, v/v) and used for the primary injection. Subsequent booster immunizations were performed every 15 days using 500 μ g of the same protein preparation emulsified in incomplete adjuvant. Antisera were collected 15 days after the injection, and tested for affinity and specificity. Antisera with high titre were pooled, and the antibody specific to azatyrosine was affinity-purified by standard procedures using a column of Sepharose containing covalently linked BSA bound to azatyrosine through its amino group. The purified antibody was eluted by pH

change, neutralized, aliquoted and stored at –20 °C. Mouse monoclonal antibodies specific to Tyr-tubulin (tubulin whose α -subunit has a C-terminal tyrosine residue; Tub1A2) and to total α -tubulin (DM1A), Protein A–peroxidase, peroxidase-conjugated rabbit anti-mouse IgG, rhodamine-conjugated goat anti-rabbit antibodies and fluorescein-conjugated goat anti-mouse secondary antibodies were from Sigma (St. Louis, MO, U.S.A.).

Cell culture

C6, COS-7, NIH 3T3, HT29 and PC12 cells were grown in Ham's F12K medium (Sigma) supplemented with 10% (v/v) FBS (Invitrogen) at 37 °C in an air/ CO_2 (19:1) incubator. Cells were plated on plastic Petri dishes (60 mm diameter) or 24-well plates at 20–40% confluence and grown for the stated periods. Culture medium was renewed every 24 h. Treatments involving cells were performed at 37 °C unless otherwise stated.

Immunoblotting and quantification of tubulin isospecies

Tubulin samples were subjected to SDS/PAGE [29], and the proteins were transferred to nitrocellulose sheets [30]. The sheets were reacted overnight at 4 °C with anti-Glu, anti-Tyr, anti-(total tubulin) or anti-Azaty antibodies (diluted 1:200, 1:1000, 1:1000 and 1:600, respectively). Sheets treated with anti-Tyr or anti-(total tubulin) were incubated for 1 h at room temperature with peroxidase-conjugated rabbit anti-mouse IgG (dilution 1:600), and then incubated for 1 h at room temperature in the presence of horseradish peroxidase conjugated to Protein A (1 μ g/ml). Colour was developed using 4-chloronaphth-1-ol. After washing, immunoblots were partially dried by pressing the sheet between tissue paper sheets and immediately scanned with a Duoscan T1200 (Agfa) connected to a PC. Absorbance values were determined using the Scion Image program. Experimental values were standardized relative to total tubulin; i.e. the absorbance of a band stained with a given antibody was divided by that of an identical sample stained with anti-(total tubulin).

Assembly and disassembly of Azaty-tubulin (tubulin α -subunit with a C-terminal azatyrosine residue instead of tyrosine)

Tyrosine (0.1 mM) and azatyrosine (1 mM) were incorporated separately into tubulin from soluble brain extracts. The preparations were filtered through Sephadex G-25 and mixed with 2 vol. of a similar soluble brain preparation that had not been used for amino acid incorporation but kept at 0 °C. To determine assembly capability, the mixtures were incubated at 37 °C under assembly conditions (0.2 mM GTP, 40% glycerol) for 30 min and then centrifuged at 100 000 g for 10 min at 27 °C. The pellet and supernatant fractions were processed for immunoblot using anti-Tyr, anti-(total tubulin) and anti-Azaty antibodies. To determine disassembly capability, pellets from parallel experiments were resuspended in the original volume with MEM buffer and kept in the cold (0 °C for 30 min) with gentle stirring. The samples were then centrifuged at 100 000 g for 10 min at 0 °C, and the pellet and supernatant fractions were processed for immunoblot as above. After immunostaining, bands corresponding to each tubulin species were quantified by densitometry. Percentage of tubulin assembly was calculated as the absorbance value of the pellet (sedimented microtubules) divided by the sum of absorbance values for pellet and supernatant, multiplied by 100. Percentage of microtubule disassembly was calculated as the absorbance value of the supernatant fraction (disassembled

tubulin) divided by the sum of absorbance values for pellet and supernatant, multiplied by 100.

Immunofluorescence

Cells cultured on coverslips were fixed with anhydrous methanol at -20°C . Samples were washed, incubated with 2% (w/v) BSA in PBS for 1 h, and stained by double indirect immunofluorescence using anti-Azatyrr and DM1A antibodies (1:600 and 1:1000 dilution in PBS containing 1% BSA, respectively). Fluorescein-conjugated anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG were used simultaneously as secondary antibodies at 1:400 and 1:800 dilution, respectively. Coverslips were mounted in FluorSave and observed for epifluorescence on an Axioplan microscope (Zeiss, Cologne, Germany).

Cell viability and proliferation

Percentage of viable cells was determined by Trypan Blue exclusion. To determine proliferation rate, cells in 96-well plates were cultured for the stated times, and cell numbers were determined in triplicate samples using Cell Titer 96 Aqueous One Solution (Promega). When indicated, the medium was replaced by 100 μl of Ham's F12K medium plus 20 μl of Cell Titer 96 Aqueous One Solution. Samples were incubated for 1 h at 37°C , and absorbance was measured at 455 nm to provide a direct estimate of cell number.

RESULTS

Incorporation of azatyrosine into the C-terminus of α -tubulin in soluble rat brain extracts

Using incubation conditions described previously for incorporation of tyrosine into tubulin of soluble rat brain extract [28], we found that azatyrosine competed with incorporation of [^{14}C]tyrosine into tubulin (Figure 1A, upper panel). Previous incubation of this extract under incorporation conditions in the presence of azatyrosine prevented subsequent incorporation of [^{14}C]tyrosine (Figure 1A, lower panel). These results suggest that azatyrosine was incorporated into the same position as [^{14}C]tyrosine, through the same enzymic system.

To determine whether azatyrosine was incorporated at the C-terminal position of α -tubulin, we established an antibody that reacts specifically with azatyrosine that has a conserved free carboxyl group (see the Materials and methods section). Resulting serum was able to recognize rat brain α -tubulin containing bound azatyrosine at its C-terminus, but not Tyr- or Glu-tubulin. Tubulin in a soluble rat brain extract that was not incubated in the presence of azatyrosine contained both Tyr- and Glu-tubulin isospecies that were not stained by anti-Azatyrr antibody (Figure 1B, lane 1). On the other hand, after incorporation of azatyrosine in the soluble fraction (Figure 1B, lane 2), intense staining was observed on a region corresponding to α -tubulin. Prior to staining with anti-Azatyrr, the nitrocellulose strip corresponding to lane 2 was reacted with anti-total α -tubulin and revealed by chemiluminescence using an Amersham Biosciences ECL[®] kit (Figure 1B, lane 3). The position of α -tubulin coincided exactly with that of the band stained with anti-Azatyrr. These results indicate that azatyrosine was bound to a protein with the same mobility as α -tubulin. To further characterize the anti-Azatyrr antibody, we ran two identical samples of soluble rat brain extract after incorporation of azatyrosine, and stained the nitrocellulose strips with anti-Azatyrr previously blocked with 500 μM tyrosine or 500 μM azatyrosine,

Figure 1 Incorporation of azatyrosine into soluble rat brain tubulin

(A) Competition and exclusion of [^{14}C]tyrosine incorporation by azatyrosine: [^{14}C]tyrosine incorporation into tubulin of soluble rat brain preparation was determined in the presence of various amounts of azatyrosine (upper panel). In a separate experiment (lower panel), incorporation of [^{14}C]tyrosine was determined as a function of incubation time, in soluble extracts that were previously incubated under incorporation conditions in the presence (○) or absence (●) of 1 mM azatyrosine and then filtered on Sephadex G-25 columns to eliminate free azatyrosine. For details see the Materials and methods section. (B) Immunoblotting determination of azatyrosine incorporated into the C-terminus of α -tubulin: Soluble rat brain extracts incubated under incorporating conditions in the absence (lane 1) or presence (lane 2) of 500 μM azatyrosine were immunoblotted and stained with anti-Azatyrr (the entire strips are shown), anti-Tyr (Tyr), anti-Glu (Glu) or anti-(total tubulin) (Total) antibodies. For Tyr, Glu and total tubulin, only the area corresponding to α -tubulin is shown. Prior to staining of the nitrocellulose strip shown in lane 2, the strip was reacted with anti-(total tubulin) and with peroxidase-labelled secondary antibody, and subsequently developed using an Amersham Biosciences ECL kit (lane 3). The arrow shows the position of α -tubulin. (C) Specific blocking by soluble azatyrosine of anti-Azatyrr immunoreactivity: the same material as in lane 2 was run in lanes 4 and 5, which were stained with anti-Azatyrr previously blocked (1 h at room temperature) with 500 μM azatyrosine and tyrosine, respectively.

respectively. Azatyrosine blocked immunoreactivity of anti-Azatyrr (Figure 1C, lane 4) whereas tyrosine had no effect (Figure 1C, lane 5). These results indicate that anti-Azatyrr is capable of discriminating between C-terminal tyrosine and azatyrosine even though these compounds differ by only one atom.

Figure 2 Kinetics of incorporation of azatyrosine into soluble rat brain tubulin

(A) Incorporation of azatyrosine as a function of incubation time as determined by immunoblot with anti-Azatyrt (upper panel) and subsequent densitometry (lower panel): only areas corresponding to tubulin band are shown. Densitometry results are expressed as absorbances ('O.D.'; arbitrary units) for samples of identical volumes. (B) Determination of K_m for azatyrosine. Samples were incubated as described in the Materials and methods section. After incubation, Azatyrt-tubulin was quantified by immunoblot with anti-(total tubulin) and anti-Azatyrt, and subsequent densitometry. Values of Azatyrt-tubulin were standardized against total tubulin as described in the Materials and methods section. Inset: reciprocal of velocity plotted against reciprocal of concentration.

Using anti-Azatyrt, we determined the incorporation of azatyrosine into tubulin as a function of incubation time. The amount of Azatyrt-tubulin increased gradually up to 60 min of incubation (Figure 2A). No staining was observed at $t = 0$, again indicating the high specificity of this antibody.

Using anti-Azatyrt to quantify Azatyrt-tubulin, we determined the apparent K_m of azatyrosine as 2.3×10^{-4} M (Figure 2B, inset), indicating that the affinity of azatyrosine for the ligase is slightly less than that of tyrosine [apparent K_m , $(2-7.5) \times 10^{-5}$ M; see [28,31]].

Together, these results indicate that azatyrosine can be incorporated into the C-terminus of the α -tubulin chain through an enzymic system similar to that for incorporation of tyrosine.

***In vitro* removal of azatyrosine from tubulin by endogenous tubulin carboxypeptidase**

The capability of tubulin carboxypeptidase to release azatyrosine from Azatyrt-tubulin was determined under conditions described previously for the release of tyrosine from Tyr-tubulin. After incorporation of azatyrosine into tubulin of a soluble rat brain extract, the preparation was incubated under microtubule assembly

Figure 3 Release of azatyrosine from Azatyrt-tubulin by tubulin carboxypeptidase

Soluble brain extract passed through a Sephadex G-25 column underwent incorporation of azatyrosine into tubulin as described in the Materials and methods section. After incubation, the preparation was passed through Sephadex G-25 to eliminate unbound azatyrosine, and the protein fraction containing Azatyrt-tubulin was collected and mixed with 3 vol. of a similar brain preparation that had been kept at 0 °C. The mixture was incubated at 37 °C under assembly conditions (0.2 mM GTP, 40 % glycerol). At the stated times, identical aliquots were removed to determine Tyr- (○) and Azatyrt-tubulin (●) by immunoblot (upper panel) and subsequent densitometry (lower panel). Values for each tubulin isospecies are expressed as percentage with respect to value at $t = 0$.

conditions, under which tubulin carboxypeptidase associates with microtubules and catalyses removal of C-terminal tyrosine [32–34]. We measured by immunoblot the amount of remaining Tyr- and Azatyrt-tubulin as a function of incubation time (Figure 3, upper panels). Densitometry of immunoblots (Figure 3, lower panel) revealed that both tubulin species decayed at the same rate while total tubulin remained constant; i.e. tubulin carboxypeptidase removes azatyrosine as efficiently as it removes tyrosine.

Capability of Azatyrt-tubulin to assemble into and to disassemble from microtubules *in vitro*

To determine the ability of Azatyrt-tubulin to form microtubules, microtubules were reconstituted from a soluble rat brain extract whose tubulin was (or was not) previously azatyrosinated (see the Materials and methods section). After separation of assembled and non-assembled tubulin fractions by centrifugation, Tyr- and Azatyrt-tubulin from both fractions were quantified. Azatyrt-tubulin assembled into microtubules as efficiently as Tyr-tubulin (Table 1). In a parallel experiment, designed to determine disassembly capability, sedimented microtubules were resuspended in

Table 1 Assembly and disassembly of Azatyr-tubulin

Percentages of assembly and disassembly were determined by quantifying each type of tubulin by immunoblot with specific antibody, and subsequent densitometry, as described in the Materials and methods section. Percentages were calculated relative to the sum of the values determined in microtubule and non-assembled fractions. Values shown are means $\pm$ S.D. from three independent experiments.

Tubulin type	Assembly (% as microtubule)	Disassembly (% as disassembled tubulin)
Azatyr-tubulin	45 $\pm$ 3	87 $\pm$ 9
Tyr-tubulin	44 $\pm$ 7	80 $\pm$ 7
Total tubulin	41 $\pm$ 6	85 $\pm$ 6

the original volume of cold buffer and kept at 0 °C for 30 min, after which the samples were centrifuged at 0 °C to separate disassembled microtubules (soluble fraction) from insoluble materials (pellet fraction). Tyr- and Azatyr-tubulin were quantified in both fractions. Microtubules were disassembled in the cold to a similar degree for both types of tubulin (Table 1).

Reversible incorporation of azatyrosine into tubulin in C6 cells

To determine whether azatyrosine can be incorporated into tubulin in living cells, glioma-derived C6 cells were used. C6 cells actively divide with a fused morphology in culture, reach confluence rapidly and die a few days later. Cells were cultured in the presence of 600 μ M azatyrosine in a medium with low amino acid content (Ham's F12K) to reduce competition by tyrosine. Cells were collected daily and analysed by immunoblot with anti-Azatyr, anti-Tyr and anti-(total tubulin) antibodies. After 2 days in culture, an intense band of Azatyr-tubulin was observed, whereas Tyr-tubulin declined from an intense band at day 0 to weak staining at day 2 (Figure 4A). These changes occurred without alteration of total tubulin (Figure 4A). Quantitative analysis of the bands on immunoblots (Figure 4B) indicated that azatyrosine present in the culture medium was significantly incorporated into tubulin in place of tyrosine at the C-terminal position. After 2 days of culture, changing the cells to an azatyrosine-free medium led to decreased Azatyr-tubulin and a corresponding increase in Tyr-tubulin, demonstrating the reversible nature of azatyrosine incorporation. During culture, the number of cells showed a complex course (Figure 4C), due mainly to low (or null) proliferation when azatyrosine was present in the medium, and acceleration after its withdrawal. Most cells remained viable during culture in the presence of azatyrosine (Figure 4C). Together, these results suggest that, after entering the cell, azatyrosine is incorporated by the ligase into the C-terminus of α -tubulin that was previously detyrosinated by tubulin carboxypeptidase. The reversible nature of this reaction is seen when cells are placed in azatyrosine-free medium; azatyrosine is removed (presumably by tubulin carboxypeptidase) and replaced by tyrosine.

We considered the possibilities that an increase of Azatyr-tubulin in cells was due not to action of the ligase but rather to *de novo* protein synthesis, and that the decrease of Azatyr-tubulin during the azatyrosine-free incubation period was due not to tubulin carboxypeptidase but rather to protein degradation. To address the first possibility, we measured incorporation of azatyrosine into tubulin by culturing cells in the presence of 600 μ M azatyrosine for 24 h in the presence and absence of protein-synthesis inhibitors. Whereas protein synthesis determined by incorporation of [35 S]methionine was lowered 95 % by the inhibitors, incorporation of azatyrosine was barely affected (Table 2,

Figure 4 Reversible incorporation of azatyrosine into tubulin in living cells

C6 cells were grown in Ham's F12K medium in the presence of 600 μ M azatyrosine. After 2 days, the medium was changed to Ham's F12K without azatyrosine. Dishes were processed at 24 h intervals to determine amounts of Azatyr-, Tyr- and total tubulin. (A) Determination by immunoblot of Azatyr-, Tyr- and total tubulin: areas corresponding only to α -tubulin are shown. (B) Amounts of Azatyr- (∇) and Tyr-tubulin ($\bullet$) as a function of time (days in culture). Values were standardized relative to total tubulin by dividing absorbance of the particular tubulin form by that of an identical sample stained with anti-(total tubulin). (C) Number of cells ($\blacksquare$, control; $\bullet$, in the presence of 600 μ M azatyrosine) and viability (∇ , in the presence of azatyrosine) determined in cultures grown in parallel and under identical conditions as in (A). For details see the Materials and methods section. O.D., absorbance

experiment 1). Incorporation of azatyrosine into tubulin was accompanied by a decrease of Tyr-tubulin (Table 2, experiment 1). These results indicate that formation of Azatyr-tubulin in cultured cells is due mainly to a post-translational reaction, presumably catalysed by tubulin tyrosine ligase, rather than to *de novo* protein synthesis. The second possibility mentioned above was also evaluated using protein-synthesis inhibitors. First, tubulin was azatyrosinated by culturing cells in the presence of azatyrosine. Then cells were changed to an azatyrosine-free medium and cultured for 24 h in the presence or absence of protein-synthesis inhibitors. The decline in Azatyr-tubulin/total tubulin ratio was similar (about 80 %) under either conditions (Table 2, experiment 2). If protein degradation had been responsible for the decline of Azatyr-tubulin this ratio would not

Table 2 Effect of protein-synthesis inhibition during the stage of incorporation of azatyrosine into tubulin, and during the stage of decline of Azatyr-tubulin after changing cells to azatyrosine-free medium

Experiment 1: to study the incorporation of azatyrosine into tubulin under inhibited protein-synthesis conditions, C6 cells were cultured for 24 h in the presence of 600 μ M azatyrosine and 10 μ Ci of [35 S]methionine (1000 Ci/mmol) in the presence or absence of protein-synthesis inhibitors (20 μ M puromycin, 20 μ M cycloheximide and 20 μ M chloramphenicol). Before ($t=0$) and after the culture period (24 h), radioactivity incorporated into protein and Tyr- and Azatyr-tubulin was quantified as described in the Materials and methods section. Experiment 2: to study the decline of azatyrosinated tubulin, C6 cells were cultured for 2 days in the presence of 600 μ M azatyrosine and then changed to azatyrosine-free medium containing 10 μ Ci of [35 S]methionine (1000 Ci/mmol). Cells were cultured for 24 h in the presence or absence of protein-synthesis inhibitors. Before ($t=0$) and after this period (24 h), radioactivity bound to protein and Tyr- and Azatyr-tubulin was determined. Values of Azatyr- and Tyr-tubulin were standardized relative to total tubulin (see the Materials and methods section).

Conditions	[35 S]Methionine in protein (c.p.m.)	Azatyr-tubulin	Tyr-tubulin
Experiment 1			
At $t=0$	0	0	1.22
At $t=24$ h			
– Inhibitors	100 000	1.24	0.80
+ Inhibitors	4500	1.30	0.82
Experiment 2			
At $t=0$	0	1.88	0.25
At $t=24$ h			
– Inhibitors	80 000	0.32	1.16
+ Inhibitors	4800	0.34	0.92

have changed (i.e. degradation of one molecule of tubulin would result in the disappearance of one molecule of Azatyr-tubulin). Furthermore, the increase of Tyr-tubulin during this stage (Table 2, experiment 2) is incompatible with a rapid protein-degradation process. Therefore, decay of Azatyr-tubulin during this stage can be attributed to the release of azatyrosine from the C-terminus of α -tubulin, probably through the action of tubulin carboxypeptidase.

The reversible incorporation of azatyrosine into tubulin in C6 cells was also observed by double immunofluorescence using anti-(total tubulin) and anti-Azatyr antibodies. Cells were grown on coverslips following the same protocol as that of Figure 4. Prior to addition of azatyrosine, staining with anti-Azatyr was negative, while that with anti-(total tubulin) showed brilliant microtubules (Figures 5A and 5B). After 2 days of culture in the presence of 600 μ M azatyrosine, the microtubule networks of all the cells were stained with anti-Azatyr. No particular subset of Azatyr-microtubules, nor any abnormality, was observed (compare Figure 5C and 5D). Enlargement of selected areas in Figures 5(C) and 5(D) showed a striking coincidence of the microtubular networks revealed by the two treatments (Figures 5C' and 5D'), indicating that tubulin containing azatyrosine can assemble into microtubules that cannot be distinguished from Tyr-tubulin microtubules, at least at this level of visualization. Two days after changing to an azatyrosine-free medium, staining of microtubules with anti-Azatyr disappeared (Figures 5E and 5F). Again, these results indicate that, in living cells, azatyrosine is incorporated reversibly into tubulin in place of tyrosine, and the ability to assemble into microtubules is not affected.

Effect of azatyrosine on C6 proliferation and morphology

In preliminary experiments, we observed that the presence of azatyrosine in culture medium prevented proliferation of C6 cells. After testing various concentrations of azatyrosine (results not

Figure 5 Microtubular network of cells grown in the presence and absence of azatyrosine

C6 cells were grown on coverslips under the protocols described for Figure 4. Samples were processed on days 0, 2 and 4 for double immunofluorescence using anti-Azatyr (A, C, E) and anti-(total tubulin) (B, D, F). The areas indicated by rectangles in (C) and (D) are enlarged in (C') and (D'), respectively. Scale bars: (E), 10 μ m; (C'), 4 μ m.

shown), for further experiments we chose 600 μ M which was the lowest concentration that stopped cell proliferation effectively. It should be noted that the antiproliferative effects of azatyrosine reported previously by other authors [23,24,35] were obtained at concentrations 4–10-fold higher than those we used. This is most likely due to the fact that we use a different culture medium containing a lower amount of tyrosine.

To study cell proliferation and morphology, C6 cells were cultured for 10 days in the absence (control) or presence of 600 μ M azatyrosine (+Azatyr) or 600 μ M azatyrosine plus 1 mM tyrosine (+Azatyr and Tyr), and for 5 additional days in azatyrosine-free medium. Cells were observed and photographed daily. We found that 4 h after plating (day 0), control and experimental cells had identical fused morphology (Figures 6A–6C). During the first 5 days of culture, cells incubated in the presence of azatyrosine did not divide (Figure 6D), whereas control cells reached confluence after 3–4 days, and by day 5 were very close and frequently superimposed (Figure 6E). By day 7, morphology of cells cultured with azatyrosine was gradually changing, and by day 10 they assumed a rounded soma with two elongated, thin processes (Figure 6G). In contrast, by day 10, post-confluent control cells started to detach from the dishes (Figure 6H) and to show pre-apoptotic signs (numerous cytoplasmic vacuoles; small rounded,

Figure 6 Effect of azatyrosine on cell division and morphology

+ Azatyr: C6 cells were plated at 20 % confluence, grown on 24-multiwell dishes in Ham's F12K medium supplemented with 600 μ M azatyrosine for 10 days and then changed to azatyrosine-free Ham's F12K medium. Control: cells grown in the absence of azatyrosine. + Azatyr and Tyr: cells grown in the presence of 600 μ M azatyrosine plus 1 mM tyrosine. On days 0, 5, 10 and 15, cells were observed by phase-contrast microscopy and photographed. Magnification, $\times 60$.

floating cells; abundant debris). After elimination of azatyrosine (day 10), cells that had been cultured in the presence of azatyrosine gradually resumed normal, fused morphology and proliferation activity, reaching confluence after a few days (Figure 6J). By this time, control cells had all detached and died. Presence of 1 mM tyrosine in culture medium containing azatyrosine blocked the anti-proliferative activity and morphology change induced by azatyrosine (Figures 6C, 6F and 6I), resulting in a pattern identical to that of the control (Figures 6B, 6E and 6H).

Azatyrosine can be incorporated into tubulin of different cell types

We investigated whether the reversible incorporation of azatyrosine into the C-terminus of α -tubulin was a phenomenon particular to C6 cells, or was common to other types of cells. We found that PC12, HT29, Cos-7 and NIH3T3 cells could all incorporate azatyrosine into tubulin at the C-terminus (Figure 7, upper panel), with a corresponding decrease in the amount of Tyr-tubulin (Figure 7, lower panel). In each of these cell types, after changing to azatyrosine-free medium (day 2), there was a decrease of Azatyr-tubulin and a corresponding increase of Tyr-tubulin.

In a separate experiment, proliferation of each of the above five cell lines stopped shortly after addition of azatyrosine to the culture medium. The cells remained viable for 2 or 3 days under these conditions, as indicated by Trypan Blue exclusion, and resumed proliferation after azatyrosine was withdrawn (results not shown).

Figure 7 Reversible incorporation of azatyrosine into tubulin in various cell lines

PC12, HT29, COS, 3T3 and C6 cells were cultured in Ham's F12K medium for 2 days in the presence of 600 μ M azatyrosine and then changed to an azatyrosine-free medium. On days 0, 2 and 4 amounts of Azatyr-tubulin (upper panel) and Tyr-tubulin (lower panel), standardized with respect to total tubulin, were measured as described in the Materials and methods section.

DISCUSSION

Although tubulin tyrosine ligase is known to be highly specific with respect to the acceptor protein tubulin, a variety of amino acids can be incorporated in place of tyrosine: phenylalanine [8,17], L-dopa [16,17], mono- and di-iodotyrosine [20], 3-fluoro-phenylalanine [18] and 3-nitro-tyrosine [15,19,21]. We demonstrated in the present study that the ligase/carboxypeptidase system operates normally when tyrosine is replaced by azatyrosine, based on several lines of *in vitro* evidence: (i) azatyrosine competed for incorporation of [14 C]tyrosine (Figure 1A, upper panel); (ii) when tubulin was used as an acceptor of azatyrosine in a first step, radioactive tyrosine was excluded from incorporation in a subsequent step (Figure 1A, lower panel); (iii) after incubation of soluble brain extract under incorporating conditions, the presence of azatyrosine bound to the C-terminus of α -tubulin was detected using an antibody specific to azatyrosine with a free carboxyl group (Figure 1B). The antibody did not react with Tyr- or Glu-tubulin (Figure 1B). Evidently, replacement of carbon-2 of the phenol group in tyrosine by nitrogen was a modification sufficient for the immunized animal to generate a specific antibody.

Azatyr-tubulin did not differ from Tyr-tubulin in its ability to act as a substrate of the detyrosinating enzyme, tubulin carboxypeptidase (Figure 3), or to assemble into microtubules *in vitro* (Table 1). However, these results do not exclude the possibility that cell functioning is altered in some way by azatyrosine.

Detection of Azatyr-tubulin in cells cultured in the presence of azatyrosine (Figures 4A and 4B) indicates that azatyrosine was able to enter the cell and to act as substrate of endogenous tubulin tyrosine ligase. Since C-terminal tyrosine is coded by most α -tubulin genes and azatyrosine could be used by tyrosyl-tRNA synthetase, the presence of azatyrosine in that position could be attributed to *de novo* protein synthesis. However, since the amount of Azatyr-tubulin in cells cultured in the presence of azatyrosine was the same under inhibited and non-inhibited protein-synthesis

conditions (Table 2), incorporation of azatyrosine into tubulin via *de novo* protein synthesis must be very low compared with that catalysed by tubulin tyrosine ligase.

When cells containing Azatyr-tubulin were changed to an azatyrosine-free medium, the amount of Azatyr-tubulin diminished abruptly (Figure 4B) while the amount of Tyr-tubulin increased. The decrease of Azatyr-tubulin appears to result from the action of tubulin carboxypeptidase rather than from protein degradation. If protein degradation were the cause, the ratio of Azatyr-tubulin to total tubulin would remain constant since both would decrease in parallel. On the contrary, as shown in Table 2, this ratio decreased greatly. Azatyrosine can enter the cell, incorporate into tubulin by the action of tubulin tyrosine ligase, and be released by tubulin carboxypeptidase. In other words, it can replace tyrosine in the tubulin tyrosination/detyrosination cycle. This conclusion is consistent with the presence of azatyrosine in microtubules as revealed by double immunofluorescence (Figure 5).

The unique ability of azatyrosine to convert a *ras*- or *c-erbB*-2-transformed phenotype to normal has been documented in numerous studies [25,35–37]. This effect was also observed in cell culture and in transgenic mice in which tumour formation was inhibited by administration of azatyrosine [38]. Studies on the mechanism of action at the molecular level showed that azatyrosine restores expression of the *rhoB* gene whose product participates in regulation of actin stress fibres [37]. It has also been demonstrated that azatyrosine substitutes for tyrosine in cellular proteins [27], since tyrosyl-tRNA synthetase does not distinguish between the two compounds. A mutant of this enzyme was developed that utilizes azatyrosine more efficiently than tyrosine [39]. It was hypothesized that azatyrosine incorporates into some proteins instead of tyrosine, giving rise to products different from those synthesized with tyrosine and thereby altering function of the transformed cell; that is, the cells stop proliferating and differentiate [23]. This hypothesis is consistent with the observation that addition of excess tyrosine to the culture medium, while inhibiting incorporation of [³H]azatyrosine into protein, precludes the ability of azatyrosine to convert the phenotype of transformed A4 cells to normal [27].

We evaluated the effect of 600 μ M azatyrosine on three gross cellular parameters: viability, proliferation and morphology. Incorporation of azatyrosine into the C-terminus of α -tubulin (Figures 4A and 4B, and Figure 5) was non-toxic to C6 cells, stopped proliferation (Figure 4C and Figure 6) and changed morphology to a more elongated shape (Figure 6). Withdrawal of azatyrosine resulted in rapid re-starting of proliferation (Figure 6). Excess tyrosine in the culture medium prevented incorporation of azatyrosine into the C-terminus of α -tubulin (results not shown), and blocked the antiproliferative and differentiating effects of 600 μ M azatyrosine on C6 cells (Figure 6). Since azatyrosine can be incorporated into the C-terminus of α -tubulin instead of tyrosine, the tyrosination/detyrosination cycle is an alternative explanation for the previously reported effects of azatyrosine as an antiproliferative agent and a revertant of malignant phenotype. However, as yet there is no direct evidence for this possibility.

Interesting clues arise from recent studies showing that Glu-tubulin accumulates in cancer cells as a consequence of suppression of tubulin tyrosine ligase [40,41], and that cells transfected with a ligase dominant negative mutant and a ligase cDNA antisense sequence have increased proliferation rates [42]. The cell cycle was blocked by microinjecting antibodies specific to Tyr-tubulin [14] and thereby altering synthesis of cyclin B. This suggests a role of the α -tubulin C-terminus in regulation of cyclin B synthesis in developing oocytes. In the context of our present results, we can hypothesize that substitution of C-terminal

tyrosine of α -tubulin by azatyrosine interferes with the cell cycle by altering the same pathway as injected antibodies.

Other phenomena characteristic of reversion of cells from transformed to normal phenotype are the adoption of a more asymmetric cell shape, and the acquisition of numerous actin stress fibres and focal adhesion sites. The small GTPase Rho is involved in these processes [43,44], and treatment with azatyrosine induces Rho expression [45]. In addition, Rho mediates microtubule stabilization and concomitant formation of detyrosinated microtubules [46]. Therefore, the ability of Rho to regulate cell shape is dependent on the status of α -tubulin C-terminus. There could be a connection between the presence of azatyrosine at the α -tubulin C-terminus and the observed change of cell shape.

Although untested, these concepts could be useful for the design of new approaches addressing the molecular mechanisms for the interesting effects of azatyrosine on cells. If these effects are related to substitution of C-terminal tyrosine by azatyrosine, it could help explain the physiological role of the cyclic tyrosination/detyrosination of tubulin.

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REFERENCES

- Barra, H. S., Arce, C. A., Rodríguez, J. A. and Caputto, R. (1974) Some common properties of the protein that incorporates tyrosine as a single unit and the microtubule protein. *Biochem. Biophys. Res. Commun.* **60**, 1384–1390.
- Barra, H. S., Arce, C. A. and Argaraña, C. E. (1988) Posttranslational tyrosination/detyrosination of tubulin. *Mol. Neurobiol.* **2**, 133–153.
- Barra, H. S., Arce, C. A. and Caputto, R. (1980) Total tubulin and its aminoacylated and non-aminoacylated forms during the development of rat brain. *Eur. J. Biochem.* **109**, 439–446.
- Arce, C. A. and Barra, H. S. (1985) Release of C-terminal tyrosine from tubulin and microtubules at steady state. *Biochem. J.* **226**, 311–317.
- MacRae, T. H. (1997) Tubulin post-translational modifications—enzymes and their mechanisms of action. *Eur. J. Biochem.* **244**, 265–278.
- Gundersen, G. G., Kalnoski, M. H. and Bulinski, J. C. (1984) Distinct populations of microtubules: tyrosinated and nontyrosinated alpha tubulin are distributed differently *in vivo*. *Cell* **38**, 779–789.
- Argaraña, C. E., Barra, H. S. and Caputto, R. (1978) Release of [¹⁴C]tyrosine from tubulinyl-[¹⁴C]tyrosine by brain extract. Separation of a carboxypeptidase from tubulin-tyrosine ligase. *Mol. Cell. Biochem.* **19**, 17–21.
- Arce, C. A., Hallak, M. E., Rodríguez, J. A., Barra, H. S. and Caputto, R. (1978) Capability of tubulin and microtubules to incorporate and to release tyrosine and phenylalanine and the effect of the incorporation of these amino acids on tubulin assembly. *J. Neurochem.* **31**, 205–210.
- Gundersen, G. G., Khawaja, S. and Bulinski, J. C. (1987) Postpolymerization detyrosination of α -tubulin: a mechanism for subcellular differentiation of microtubules. *J. Cell Biol.* **105**, 251–264.
- Murofushi, H. (1980) Purification and characterization of tubulin-tyrosine ligase from porcine brain. *J. Biochem. (Tokyo)* **87**, 979–984.
- Schroder, H. C., Wehland, J. and Weber, K. (1985) Purification of brain tubulin-tyrosine ligase by biochemical and immunological methods. *J. Cell Biol.* **100**, 276–281.
- Wehland, J., Schroder, H. C. and Weber, K. (1986) Isolation and purification of tubulin tyrosine ligase. *Methods Enzymol.* **134**, 170–179.
- Ersfeld, K., Wehland, J., Plessmann, U., Dodemont, H., Gerke, V. and Weber, K. (1993) Characterization of the tubulin-tyrosine ligase. *J. Cell Biol.* **120**, 725–732.
- Vée, S., Lafanechère, L., Fisher, D., Wehland, J., Job, D. and Picard, A. (2001) Evidence for a role of the α -tubulin C terminus in the regulation of cyclin B synthesis in developing oocytes. *J. Cell Sci.* **114**, 887–898.
- Chang, W., Webster, D. R., Salam, A. A., Gruber, D., Prasad, A., Eiserich, J. P. and Bulinski, J. C. (2002) Alteration of the C-terminal amino acid of tubulin specifically inhibits myogenic differentiation. *J. Biol. Chem.* **277**, 30690–30698.

- 16 Rodríguez, J. A., Barra, H. S., Arce, C. A., Hallak, M. E. and Caputto, R. (1975) The reciprocal exclusion by L-Dopa (L-3,4-dihydroxyphenylalanine) and L-tyrosine of their incorporation as single units into a soluble rat brain protein. *Biochem. J.* **149**, 115–121
- 17 Arce, C. A., Rodríguez, J. A., Barra, H. S. and Caputto, R. (1975) Incorporation of L-tyrosine, L-phenylalanine and L-3,4-dihydroxyphenylalanine as single units into rat brain tubulin. *Eur. J. Biochem.* **59**, 145–149
- 18 Monasterio, O., Nova, E., López-Brauet, A. and Lagos, R. (1995) Tubulin-tyrosine ligase catalyzes covalent binding of 3-fluoro-tyrosine to tubulin: kinetic and [¹⁹F]NMR studies. *FEBS Lett.* **374**, 165–168
- 19 Eiserich, J. P., Estévez, A. G., Bamberg, T. V., Ye, Y. Z., Chumley, P. H., Beckman, J. S. and Freeman, B. A. (1999) Microtubule dysfunction by posttranslational nitrotyrosination of alpha-tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6365–6370
- 20 Joniau, M., Coudijzer, K. and De Cuyper, M. (1990) Reaction of alpha-tubulin with iodotyrosines catalyzed by tubulin:tyrosine ligase: carboxy-terminal labeling of tubulin with [¹²⁵I]moniodotyrosine. *Anal. Biochem.* **184**, 325–329
- 21 Bisig, C. G., Purro, S. A., Contín, M. A., Barra, H. S. and Arce, C. A. (2002) Incorporation of 3-nitrotyrosine into the C-terminus of α -tubulin is reversible and not detrimental to dividing cells. *Eur. J. Biochem.* **269**, 5037–5045
- 22 Inouye, S., Shomura, T., Tsuruoka, T., Ogawa, Y., Watanabe, H., Yoshida, J. and Niida, T. (1975) L- β -(5-hydroxy-2-pyridyl)-Alanine and L- β -(3-hydroxyureido)-alanine from *Streptomyces*. *Chem. Pharm. Bull.* **23**, 2669–2677
- 23 Shindo-Okada, N., Makabe, O., Nagahara, H. and Nishimura, S. (1989) Permanent conversion of mouse and human cells transformed by activated ras or raf genes to apparently normal cells by treatment with the antibiotic azatyrosine. *Mol. Carcinog.* **2**, 159–167
- 24 Monden, Y., Shindo-Okada, N. and Nishimura, S. (1998) Azatyrosine: mechanism of action. In *G Proteins, Cytoskeleton and Cancer* (Maruta, H. and Kohama, K., eds.), pp. 317–325, R.G. Landes Company, Austin, TX
- 25 Fujita-Yoshigaki, J., Yokoyama, S., Shindo-Okada, N. and Nishimura, S. (1992) Azatyrosine inhibits neurite outgrowth of PC12 cells induced by oncogenic Ras. *Oncogene* **7**, 2019–2024
- 26 Chung, D. L., Brandt-Rauf, P. W., Weinstein, I. B., Nishimura, S., Yamaizumi, Z., Murphy, R. B. and Pincus, M. R. (1992) Evidence that the ras oncogene-encoded p21 protein induces oocyte maturation via activation of protein kinase C. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1993–1996
- 27 Monden, Y., Nakamura, T., Kojiri, K., Shindo-Okada, N. and Nishimura, S. (1996) Azatyrosine is incorporated into proteins instead of tyrosine residues with the resultant conversion of transformed cells with a normal phenotype. *Oncol. Rep.* **3**, 625–629
- 28 Barra, H. S., Rodríguez, J. A., Arce, C. A. and Caputto, R. (1973) A soluble preparation from rat brain that incorporates into its own proteins [¹⁴C]arginine by a ribonuclease-sensitive system and [¹⁴C]tyrosine by a ribonuclease-insensitive system. *J. Neurochem.* **48**, 97–108
- 29 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 30 Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- 31 Barra, H. S., Arce, C. A., Rodríguez, J. A. and Caputto, R. (1973) Incorporation of phenylalanine as a single unit into rat brain protein: reciprocal inhibition by phenylalanine and tyrosine of their respective incorporations. *J. Neurochem.* **21**, 1241–1251
- 32 Arce, C. A. and Barra, H. S. (1983) Association of tubulin-tyrosine carboxypeptidase with microtubules. *FEBS Lett.* **157**, 75–78
- 33 Sironi, J. J., Barra, H. S. and Arce, C. A. (1997) Association of tubulin carboxypeptidase activity with microtubules in brain extracts is modulated by phosphorylation/dephosphorylation processes. *Mol. Cell. Biochem.* **170**, 9–16
- 34 Contín, M. A., Sironi, J. J., Barra, H. S. and Arce, C. A. (1999) Association of tubulin carboxypeptidase with microtubules in living cells. *Biochem. J.* **339**, 463–471
- 35 Monden, Y., Hamano-Takaku, F., Shindo-Okada, N. and Nishimura, S. (2000) Azatyrosine. Mechanism of action for conversion of transformed phenotype to normal. *Ann. N.Y. Acad. Sci.* **886**, 109–121
- 36 Monden, Y., Nakamura, T., Kojiri, K., Shindo-Okada, N. and Nishimura, S. (1996) Azatyrosine inhibits the activation of c-Raf-a, c-Jun and AP1 but not the activation of Ras during signal transduction triggered by oncogenic c-ErbB-2. *Oncol. Rep.* **3**, 33–40
- 37 Monden, Y., Nakamura, T., Kojiri, K., Shindo-Okada, N. and Nishimura, S. (1996) Stimulation of the expression of rhoB contributes to the ability of azatyrosine to convert c-ErbB-2 transformed cells to normal morphology. *Oncol. Rep.* **3**, 49–55
- 38 Izawa, M., Takayama, S., Shindo-Okada, N., Doi, S., Kitamura, M., Katsuki, M. and Nishimura, S. (1992) Inhibition of chemical carcinogenesis *in vivo* by azatyrosine. *Cancer Res.* **52**, 1628–1630
- 39 Hamano-Takaku, F., Iwama, T., Saito-Yano, S., Takaku, K., Monden, Y., Kitabatake, M., Soll, D. and Nishimura, S. (2000) A mutant *Escherichia coli* tyrosyl-tRNA synthetase utilizes the unnatural amino acid azatyrosine more efficiently than tyrosine. *J. Biol. Chem.* **275**, 40324–40328
- 40 Lafanechère, L., Courtay-Cahen, C., Kawakami, T., Jacrot, M., Rüdiger, M., Wehland, J., Job, D. and Margolis, R. L. (1998) Suppression of tubulin tyrosine ligase during tumor growth. *J. Cell Sci.* **111**, 171–181
- 41 Mialhe, A., Lafanechère, L., Treilleux, I., Peloux, N., Dumontet, C., Brémond, A., Panh, M.-H., Payan, R., Wehland, J., Margolis, R. L. and Job, D. (2001) Tubulin detyrosination is a frequent occurrence in breast cancers of poor prognosis. *Cancer Res.* **61**, 5024–5027
- 42 Mas, C. R., Arregui, C. O., Filiberti, A., Argaraña, C. E. and Barra, H. S. (2002) Cloning of rat olfactory bulb tubulin tyrosine ligase cDNA: a dominant negative mutant and an antisense cDNA increase the proliferation rate of cells in culture. *Neurochem. Res.* **27**, 1445–1450
- 43 Ridley, A. J. and Hall, A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389–399
- 44 Miura, Y., Kikuchi, A., Musha, T., Kuroka, S., Yaku, H., Sasaki, T. and Takai, Y. (1993) Regulation of morphology by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI) in Swiss 3T3 cells. *J. Biol. Chem.* **268**, 510–515
- 45 Krzyzosiak, W. J., Shindo-Okada, N., Teshima, H., Nakajima, K. and Nishimura, S. (1992) Isolation of genes specifically expressed in flat revertant cells derived from activated ras-transformed NIH 3T3 cells by treatment with azatyrosine. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4879–4883
- 46 Cook, T. A., Nagasaki, T. and Gundersen, G. G. (1998) Rho guanosine triphosphate mediates the selective stabilization of microtubules induced by lysophosphatidic acid. *J. Cell Biol.* **141**, 175–185

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