Posttranslational Tyrosination/Detyrosination of Tubulin

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Abstract

Tubulin can be posttranslationally modified at the carboxyl terminus of the α-subunit by the addition or release of a tyrosine residue. These reactions involve two enzymes, tubulin: tyrosine ligase and tubulin carboxypeptidase. The tyrosine incorporation reaction has been described mainly in nervous tissue but it has also been found in a great variety of tissues and different species. Molecular aspects of the reactions catalyzed by these enzymes are at present well known, especially the reaction carried out by the ligase. Several lines of evidence indicate that assembled tubulin is the preferred substrate of the carboxypeptidase, whereas nonassembled tubulin is preferred by the ligase. Apparently this posttranslational modification does not affect the capacity of tubulin to form microtubules but it generates microtubules with different degrees of tyrosination. Variation in the content of the carboxyterminal tyrosine of α-tubulin as well as changes in the activity of the ligase and the carboxypeptidase are manifested during development. Changes in the cellular microtubular network modify the turnover of the carboxyterminal tyrosine of α-tubulin. Different subsets of microtubules with different degrees of tyrosination have been detected in interphase cells and during the mitotic cycle. Data from biochemical, immunological, and genetic studies have been compiled in this review; these are presented, with pertinent comments, with the hope of facilitating the comprehension of this particular aspect of the microtubule field.

Index Entries: Tyrosination; detyrosination; tubulin; microtubules; tyrosination/detyrosination of tubulin; posttranslational modification of tubulin; carboxyl terminus of a-tubulin; state of tyrosination of the carboxyl terminus of α-tubulin; cytoskeleton.

Introduction

Microtubules are cytoskeletal filaments present in all eukaryotic cells and are involved in a variety of cellular functions including mitosis, cell motility, maintenance of cell shape, intracellular transport, and secretion. In the nervous system, microtubules are also involved in neurite extension and axoplasmic transport. The major protein component of microtubules is tubulin, a heterodimer composed of an α and a β subunit, each with a molecular weight of approximately 55 Kd. In addition to tubulin, several microtubule-associated proteins (MAPs) have been identified in neural tissue. These include high molecular weight proteins (MAP 1 and MAP 2) and tau proteins. Biochemical and functional aspects of microtubules have previously been reviewed (Roberts and Hyams, 1979; Soifer, 1986).

Based on the apparent functional and biochemical diversity of microtubules, many different mechanisms are hypothesized to regulate and determine their functions. Analysis of tubulin shows that this protein is not composed of unique α and β polypeptides, but of a heterogeneous mixture of slightly different subunits (Feit et al., 1970; Gozes and Littauer, 1978; Lee et al., 1986), which arise as the products of different genes and from posttranslational modifications. The transcriptional turn-on and -off of the different α- and β-tubulin genes is thought to establish more or less stable functional changes during development, such as changes occurring during organogenesis. On the other hand, posttranslational modifications may constitute a fast, transient mechanism by which the function of microtubules can be modified in response to a sudden intracellular change.

Several possible mechanisms by which the functions of tubulin or microtubules could be regulated have been suggested. These include the interactions with MAPs (Borisy et al., 1974; Dentler et al., 1975; Weingarten et al., 1975); the binding of GTP (Jacobs, 1979), Ca^{2+} (Solomon, 1977), and calmodulin (Marcum et al., 1978; Ku-

magai and Nishida, 1980); the phosphorylation of serine residues on β-tubulin (Eipper, 1972; Gard and Kirschner, 1985), and the acetylation of lysine residues on α-tubulin (Piperno and Fuller, 1985; L'Hernault and Rosenbaum, 1985a,b). In addition to the interactions and posttranslational modifications mentioned, it was found that the carboxyl terminus of α-tubulin undergoes a reversible posttranslational tyrosination/detyrosination. This finding stems from studies in this laboratory. Initially, we showed that the addition of [^{14}C]-tyrosine to a soluble preparation from rat brain led to the tyrosination of a single endogenous protein (Barra et al., 1972, 1973a, 1974). The reaction was tRNA-independent and the cofactors required were found to be ATP, Mg^{2+}, and K^+. We also described the properties of the acceptor protein, which was identified as tubulin (Barra et al., 1974). On the other hand, digestion with pancreatic carboxypeptidase A indicated that the radioactive amino acid was incorporated into the carboxyl terminus of the acceptor protein (Barra et al., 1973b). Analysis of small radioactive peptides obtained by partial acid hydrolysis showed that the amino acid was incorporated through a peptide bond on the α-carboxyl of glutamic acid or glutamine (Arce et al., 1975a) of the α-tubulin chain (Arce et al., 1975b). At present, it is an established fact that the new peptide bond is made on a glutamic acid residue.

The incorporated tyrosine can be removed by two different enzymatic reactions. One requires the presence of ADP and inorganic phosphate and is catalyzed by the reverse action of tubulin: tyrosine ligase (Rodríguez et al., 1973; Raybin and Flavin, 1977a). The other reaction is catalyzed by a carboxypeptidase that seems to be specific for tubulin and therefore we named it tubulin carboxypeptidase (Hallak et al., 1977; Argarañа et al., 1978,1980). These reactions are shown schematically in Fig. 1.

The physiological role of this tyrosination/detyrosination reaction is at present unknown. Under in vitro conditions tyrosinated or detyrosinated tubulin assembles equally well into microtubules. However, it is possible that this posttranslational modification plays a regulatory role in microtubule formation or in some function carried out by these structures in the cell.

The aim of this review is to describe what is known at present about the enzymes involved in the tyrosination/detyrosination reaction, the turnover of the carboxyterminal tyrosine, the factors that determine the tyrosination state of microtubules, and the genetics of tubulin in relation with the presence of tyrosine at the carboxyl terminus of the α-chain. Some aspects of these topics have been reviewed previously (Thompson, 1982).

Tubulin:Tyrosine Ligase

Specificity

Besides tyrosine (Km = 0.02 m*M*), tubulin : tyrosine ligase can also catalyze in vitro the addition of phenylalanine (Km = 1.2 m*M*) and 3,4-dihydroxyphenylalanine (DOPA) (Km = 0.16–1.2 m*M*) at the same position of tyrosine (Barra et al., 1973b; Rodríguez et al., 1975; Arce et al., 1975b). These findings have been confirmed by Deanin and Gordon (1976). The requirement for cofactors is similar for the three amino acids: ATP (Km = 0.75 m*M*), Mg^{2+} (optimal concentration 8–16 m*M*), and K^+ (optimal concentration >30 m*M*). Considering the concentration of these aromatic amino acids in brain (Agrawal et al., 1966; Bayer and McMurray, 1967; Johnson et al., 1973) and their relative affinities for tubulin, it was predicted that in vivo the amount of tyrosine in the carboxyl terminus of tubulin should be much greater than the amount of phenylalanine and, in turn, this amount should be higher than that of DOPA (Rodríguez et al., 1975). As far as we know there is no report describing the presence of DOPA in

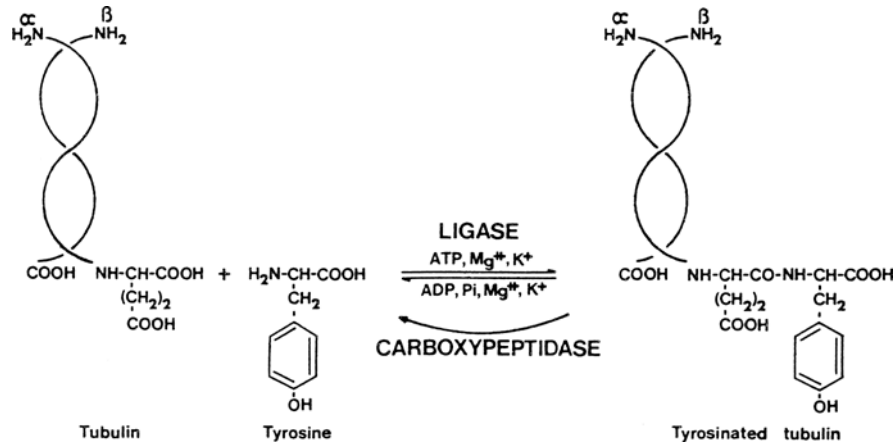

Fig. 1. Tyrosination/detyrosination of the α-chain of tubulin.

native tubulin. Most tubulin molecules contain tyrosine, whereas phenylanine is present only in very low proportions (Rodríguez and Borisy, 1978,1979b).

As mentioned above, the incorporated tyrosine can be removed by the reverse action of the ligase. Nath and Flavin (1984) reported that after tyrosination in a mince from chick brain, the isolated tubulin could not be detyrosinated by the ligase; however, when tubulin was tyrosinated in the soluble brain extract it was readily detyrosinated. We studied this problem in rat brain tissue but could not obtain evidence for any difference between the tubulin labeled in soluble extracts and brain slices. Both tubulins were detyrosinated by the ligase with similar kinetics (Barra et al., 1982).

Studies performed in vitro and in vivo indicate that a-tubulin is the only substrate of the ligase. When rat brain extract was incubated with [^{14}C]tyrosine and then fractionated by electrophoresis on SDS-gels, the only radioactive protein corresponded to the a-chain of tubulin (Arce et al., 1975b; Argaraña et al., 1977). Raybin and Flavin (1977a), using purified enzyme, tested the acceptor capacity of other proteins containing carboxyterminal glutamate or glutamine. They did not find incorporation of tyrosine into horse heart cytochrome C and avidin (carboxyterminal glutamate), or micrococcal nuclease and apolipoprotein-glutamine (carboxyterminal glutamine).

It has been established that only a fraction of the tubulin pool is substrate for the ligase. The proportion of the tyrosinable tubulin pool changes from one tissue to another and also with the development of the animal (Rodríguez and Borisy, 1978; Barra et al., 1980; Kobayashi and Matsumoto, 1982). We have no information on structural differences between tyrosinable and nontyrosinable forms of tubulin. Whether these differences arise from a modification in the primary structure of either α- or β-tubulin or is owing to a posttranslational modification of the protein remains to be elucidated. This aspect will be further discussed in another section.

Nonassembled Tubulin vs Microtubules as Substrate for the Ligase

What is the influence of the assembly state of tubulin on the tyrosine incorporating reaction? In vitro studies have shown that only nonassembled tubulin is the substrate for the ligase (Arce et al., 1978). When short term [^{14}C]tyrosine incorporation was carried out under conditions in which the formation of microtubules was in plateau, the incorporation oc-

curred mainly on nonassembled tubulin. Only a low proportion of the labeled tyrosinated tubulin was found in microtubules and this was attributed to an exchange of tubulin between microtubules and the nonassembled tubulin pool. The lack of incorporation of [^{14}C]tyrosine into microtubules is owing to some impediment arising from tubulin being into a complex structure, since after separation and disassembly of microtubules, [^{14}C]tyrosine can be incorporated in a higher proportion than that obtained in the originally nonassembled pool (Arce et al., 1978). A similar conclusion was recently obtained using mature chicken erythrocytes (Beltramo et al., 1987b). In these cells it was found that the incorporation occurred almost exclusively into the nonassembled tubulin pool. The marginal band was virtually devoid of incorporated tyrosine; however, this was not owing to the lack of tubulin with tyrosine-acceptor capacity. In brain slices, contrary to what was found in soluble extracts or chicken erythrocytes, microtubules are tyrosinated to a greater extent than the nonassembled tubulin pool (Beltramo et al., 1987a). These results, in addition to the finding that the ligase is isolated as a soluble entity from erythrocytes (Beltramo et al., 1987b), and associated with microtubules from brain slices (Beltramo et al., 1987a) suggest that in vivo the ligase acts preferentially on tubulin or microtubules depending on the nature of the tissue. Nevertheless, it is equally likely that in every tissue the incorporation occurs exclusively on nonassembled tubulin; the high labeling of microtubules in brain slices could be explained as incorporation of [^{14}C]tyrosine into a small pool of nonassembled tubulin that is readily assembled into microtubules.

The preference of the ligase for nonassembled tubulin was also demonstrated in cultured cells (Gundersen et al., 1987; Webster et al., 1987; Bré et al., 1987). Treatment with nocodazole produced the disassembly of the microtubule network and the transformation of detyrosinated tubulin to the tyrosinated form.

Isolation and Properties

Raybin and Flavin (1975) obtained a partially purified tubulin:tyrosine ligase preparation and found that the enzyme has a molecular weight of 35 Kd. The ligase associates with tubulin dimers forming a stoichiometric 1 : 1 complex of 150 Kd (Raybin and Flavin, 1977b; Pierce et al., 1978), but not with microtubules when these are formed in vitro (Raybin and Flavin, 1975; Arce and Barra, 1983). Using DEAE-cellulose chromatography, Sepharose-sebacic acid hydrazide-ATP affinity chromatography and Sepharose-tubulin affinity chromatography, Murofushi (1980) obtained an enzyme preparation that rendered a single band as analyzed by polyacrylamide gel electophoresis. The amount of the enzyme that can be obtained by this procedure is small and the enzyme rather unstable. More recently an immunological method was developed that has made it possible to obtain milligram quantities of the ligase (Schroeder et al., 1985). The preparation may be stabilized by adding glycerol.

Novel information on the interaction between the ligase and tubulin has been recently reported by Wehland and Weber (1987) who developed several monoclonal antibodies to the ligase. They confirmed the 1:1 complex with the tubulin dimer. The stable complex involves a ligase-binding site which is located on the b -tubulin subunit. The monoclonal antibody YL 1/2 (Kilmartin et al., 1982), which is specific for tyrosinated tubulin (Wehland et al., 1983) binds to the carboxyl end of the a-subunit. The binding of this antibody or the removal of the carboxy-terminal region of both subunits by treatment with subtilisin, does not affect the formation of the complex (Wehland and Weber, 1987). Controlled digestion of the ligase with protease V8 produced two fragments of 14 and 30 Kd. One of the monoclonal antibodies (LA/C4), which specifically recognizes the 30-Kd fragment, allowed Wehland and Weber to establish that this fragment contains the binding site for b-tubulin

and ATP. Monoclonal antibody LA/C4 was found not to inhibit the incorporation of tyrosine by the ligase. Another monoclonal antibody (ID3), which inhibits the incorporation of tyrosine, reacts specifically with the 14-Kd fragment, suggesting that the ligase catalytic domain is located in this region.

Tissue Distribution and Activity During Development

Initially we believed that tubulin:tyrosine ligase was present exclusively in nervous tissue; however, soon after it was shown to be present in extracts of every avian and mammalian tissue so far examined, with the level of activity much higher in brain than in any other tissue tested (Deanin and Gordon, 1976; Raybin and Flavin, 1977b). The ubiquity of this enzyme was further shown by the activity found in neural tissue of representative organisms from several other major vertebrate classes (chondrichthyes through reptiles) (Preston et al., 1979), in cultured neuroblastoma-glioma hybrid cells (Nath and Flavin, 1979), HeLa cells (Bulinski et al., 1980), myoblast cells (Thompson et al., 1979), human polymorphonuclear leukocytes (Nath et al., 1982), amphibian oocytes (Preston et al., 1981; Barra et al., 1987), sea urchin eggs (Kobayashi and Flavin, 1981), and *Caenorhabditis elegans* (Gabius et al., 1983). The enzyme was not detected, however, in several invertebrate species (Preston et al., 1979).

The developmental pattern of tubulin:tyrosine ligase activity has been described in different tissues in the chick. In brain the ligase activity exhibited its highest value around d 14–16 of the embryo (Deanin et al., 1977; Rodríguez and Borisy, 1978). The increase of the enzyme activity was found to be temporally correlated with an increase of the proportion of tyrosinated tubulin (Rodríguez and Borisy, 1978). In the thigh muscle there was a sharp peak in enzyme activity at d 13 (Deanin et al., 1977) when, in this culture system, myotube formation was proceeding rapidly. In the dorsal root ganglion, the most rapid rise in ligase activity found corresponds to the onset of the formation of the processes (Pierce et al., 1978).

On the other side, the activity of ligase through the cell cycle was determined in two Chinese hamster cells, V79 and CHO (Forrest and Klevecz, 1978). In V79 cells the enzyme shows two peaks of activity that corrrespond to the early S and mid to late S phases of the cell cycle. CHO cells display a major peak of activity just before mid-S phase.

In *Xenopus laevis*, tubulin : tyrosine ligase is present through oogenesis and its activity does not change until 34 h post-fertilization (Preston et al., 1981).

Tubulin Carboxypeptidase

The tubulin carboxypeptidase activity present in soluble brain extracts was first reported by Hallak et al. (1977), who noted that the [^{14}C]tyrosine incorporated in vitro into tubulin was partially lost during incubations of [^{14}C]tyrosine-labeled tubulin with extracts. The releasing activity varied according to microtubule assembly conditions: The higher the amount of microtubules formed, the higher the releasing activity. The presence of a tyrosine-releasing activity in brain supernates was later confirmed by Thompson (1977). Even though the work by Hallak et al. suggested that the releasing activity was not owing to the reverse action of the ligase but to a distinct enzyme, the results were not conclusive. Argaraña et al. (1978) separated two tyrosine releasing activities, one owing to tubulin:tyrosine ligase and other with the characteristics of a carboxypeptidase. The latter enzyme, which we first named tubulinyl-tyrosine carboxypeptidase, was partially purified and some properties described (Argaraña et al., 1980). Other names have been used for this enzyme: carboxypeptide tubulin

(Kumar and Flavin, 1981), tyrosinotubulin carboxypeptidase (Martensen, 1982), and tubulin carboxypeptidase (Modesti and Barra, 1986). In this review we use this last name.

Isolation

Argaraña et al. (1980) used pH fractionation, precipitation with ammonium sulfate, and chromatography on CM-Sephadex to partially purify the carboxypeptidase from chicken brain extracts. The specific activity of enzyme was increased about 250-fold compared to the starting material. Slight modifications of the method allowed us to increase the purification to 470-fold (Modesti et al., 1984).

Tubulin carboxypeptidase was also purified from bovine brain extracts by precipitation with ammonium sulfate, DEAE-cellulose, and CM-cellulose chromatography (Kumar and Flavin, 1981; Martensen, 1982). Instability of the partially purified enzyme could be reduced by storage at -80°C as a concentrated solution.

Properties

According to its behavior on Sephadex G-200, the enzyme has a mol wt of about 90 Kd (Argaraña et al., 1980). By several criteria the enzyme was shown to be different from carboxypeptidase A (Argaraña et al., 1980).

Tubulin carboxypeptidase catalyzes the release of carboxy-terminal tyrosine from tyrosinated tubulin without affecting the remaining polypeptide as a potential substrate for the ligase. After detyrosination by tubulin carboxypeptidase, tubulin was shown to be capable of accepting [^{14}C]tyrosine again (Hallak et al., 1977).

Tubulin containing carboxyterminal phenylalanine on its α-chain may also serve as substrate for the carboxypeptidase (Arce et al., 1978). From the percentage of inhibition produced by several *N*-blocked dipeptides on the release of [^{14}C]tyrosine, it was suggested that the enzyme acts preferentially on dipeptides with identical sequences to the α-tubulin carboxyl terminus, that is, Glu-Tyr and Glu-Phe (Argaraña et al., 1980).

Activators

An early observation was that Mg^{2+} stimulated the reaction catalyzed by tubulin carboxypeptidase from rat (Argaraña et al., 1982) or chicken brain (Argaraña et al., 1980; Barra et al., 1982). Therefore, this ion is routinely added to the incubation system used for the assay of tubulin carboxypeptidase.

In the absence of Mg^{2+}, the polyamines spermine, spermidine, and putrescine also activated the releasing reaction with an optimum at 0.06, 1, and 6m*M*, respectively (Barra and Argaraña, 1982). When Mg^{2+} was present at optimal concentration (2–4 m*M*), the polyamines had an inhibitory effect.

Inhibitors

Under assembly conditions, the rate of tyrosine release from tyrosinated tubulin has been shown to be diminished by some compounds that were assumed to be inhibitors of the carboxypeptidase. For instance, colchicine reduced the releasing activity to about one-fourth (Hallak et al., 1977). However, this effect was demonstrated to be owing to the lack of microtubule formation rather than to a direct action of colchicine on the enzyme (Arce and Barra, 1985). To avoid this kind of ambiguity, the carboxypeptidase was assayed in a system in which the substrate, [^{14}C]tyrosinated tubulin, was in the nonasembled state. By incubation in this system, Argaraña et al. (1981) found two inhibitors in soluble brain extracts that were identified as proteoglycans and soluble RNA. Endogenous proteins of basic nature also showed inhibitory activity (Modesti et al., 1984).

In addition, several polyanions such as heparin, chondroitin sulfate, polyadenylic acid, and polyglutamic acid, and several polycations such as polylysine, protamine, and myelin basic protein showed inhibitory activity (Modesti and Barra, 1986; López et al., 1987). Since the carboxypeptidase possesses basic properties the inhibition may be explained by electrostatic interactions between the enzyme and polyanions. The inhibitory effect of polycations seems to be owing to the interaction between these compounds and the substrate tubulin through the formation of amorphous aggregates (Modesti and Barra, 1986; López et al., 1987). The binding of an endogenous brain protein to tubulin leading to the formation of an insoluble complex had been previously described by Kumar and Flavin (1982b).

Nonassembled Tubulin vs Microtubules as Substrates for Tubulin Caboxypeptidase

The fact that the tyrosine-releasing activity in brain extracts was higher in conditions favoring assembly of microtubules (Hallak et al., 1977) suggested the possibility that microtubules could be better substrates than nonassembled tubulin. This possibility was supported by results from several laboratories. Thompson (1977) found that after assembly the amount of [^{14}C]tyrosine bound to tubulin in microtubules was lower than that in nonassembled tubulin. By incubating fractions containing nonassembled [^{14}C]tyrosinated tubulin and [^{14}C]tyrosinated microtubules we found (Arce et al., 1978) a higher releasing activity in the microtubule fraction. Using purified carboxypeptidase, Kumar and Flavin (1981) showed that tubulin purified by assembly-disassembly was detyrosinated 2–3-fold faster than assembly-incompetent tubulin (MAPs-depleted). They also found that when assembly-incompetent tubulin was induced to assemble by the addition of MAPs or taxol, the rate of tyrosination was increased 3–5-fold. Endogenous tubulin carboxypeptidase present in cycle-purified microtubule protein preparations was also shown to prefer microtubules as substrate (Arce and Barra, 1985); under assembly conditions the tyrosine releasing activity was 2–3-fold higher than in the same system containing colchicine (which *per se* has no effect on the enzyme).

Even though microtubules appear to be the preferred substrate for the carboxypeptidase, nonassembled tubulin can also serve as substrate (Kumar and Flavin, 1981; Arce and Barra, 1985). We have tested the effect of several compounds that interact with tubulin and different conditions of incubation on the rate of tyrosine release from nonassembled tubulin. In no case was the releasing activity similar or higher than that obtained with microtubules under standard conditions of incubation (Beltramo et al., 1986). This result in addition to the fact that the carboxypeptidase associates with microtubules (Arce and Barra, 1983) and that detyrosination occurs mainly on microtubules in a system in which tubulin and microtubules are at steady state (Arce and Barra, 1985), indicates that microtubules may be the exclusive substrate for the carboxypeptidase under physiological conditions. Studies with cultured muscle cells revealed that the turnover of the carboxyterminal tyrosine of α-tubulin is dependent on the presence of intact microtubules (Thompson et al., 1979). This result might be explained accepting that the carboxypeptidase is associated and acting mainly on microtubules and providing, by exchange, detyrosinated tubulin to the nonassembled pool that could be then retyrosinated by tubulin:tyrosine ligase. This view fits quite well with recent findings obtained by Gundersen et al. (1987) who used antibodies specific to tyrosinated and detyrosinated tubulin and double immonfluorescence to reveal the cellular microtubular array. When microtubules of TC-7 cells were allowed to reform after complete depolymerization, initially only tyrosinated

microtubules were detected. Detyrosinated microtubules reappeared with a delay of about 25 min. Treatment with taxol, azide, or vinblastine to stabilize polymeric tubulin, all resulted in time-dependent increases in polymeric detyrosinated tubulin levels, further supporting the hypothesis of post-polymerization detyrosination. These investigators also found that when microtubules were induced to de- polymerize, no detyrosinated tubulin was detected, indicating that it was retyrosinated in the nonassembled state. Studies carried out on Madin-Darby canine cells (Bré et al., 1987), on CHO and 3T3 cells (Wehland and Weber, 1987), and on *Trypanosoma brucei* (Sherwin et al., 1987) led to similar conclusions.

Turnover of the Carboxyterminal Tyrosine

The first evidence of the occurrence of the tyrosination of tubulin in vivo was reported in 1977 by Argaraña et al. (1977) who demonstrated the incorporation of [^{14}C]tyrosine into the carboxyl terminus of α-tubulin by injecting the radioactive tyrosine into rat brain under conditions in which protein synthesis was inhibited. This posttraslational modification of tubulin was also demonstrated to occur in myogenic cells in culture (Thompson, 1977). The incorporation of labeled tyrosine into tubulin was assumed to be owing to exchange between incorporated and free tyrosine rather than occupancy of free sites. Thompson et al. (1979) demonstrated that the turnover of the carboxyterminal tyrosine of the α-tubulin in myogenic cells in culture is very rapid compared to the turnover of tubulin (half-lives: 40 min and 2 d, respectively) and that this rapid turnover is dependent on the presence of intact microtubules.

In several later studies, changes in the turnover were shown to occur concomitantly with modifications of the physiological status of the cell. Deanin et al. (1981) showed that CHO cells maintained in their epithelial-like form do not incorporate tyrosine post-translationally, whereas preincubation with dibutyryl cyclic AMP plus testosterone, which leads to a change in cell shape, activates the tyosination reaction. Changes in cell shape, which involve disintegration and reformation of microtubules, would generate tyrosinable substrate for the ligase.

Incubation of leukocytes with the chemoattractant formyl-methionyl-leucyl-phenylalanine (FMLP) induces locomotion with corresponding cytoskeletal rearrangement. Motile leukocytes showed stimulated tubulin tyrosination in comparison with control leukocytes (not treated with FMLP) (Nath et al., 1981). Again, this result indicated a correlation between reorganization of microtubules and a higher amount of tyrosinable substrate. FMLP also stimulated the tyrosination of tubulin in human polymorphonuclear leukocytes (Nath et al., 1982). This stimulation is dependent on the presence of extracellular Ca^{2+} and the Ca^{2+}-ionophore A23187 (Nath and Gallin, 1986). The diminished stimulation caused by the addition of ascorbate or other reducing agents suggested a possible relationship between cellular redox state and tubulin tyrosination (Nath et al., 1982).

During nerve growth factor-induced regeneration of neurites from PC 12 cells the incorporation of [^{14}C]tyrosine into the carboxyl terminus of α-tubulin was stimulated in comparison with control cells (Deanin et al., 1982). Furthermore, the incorporation of [^{14}C]tyrosine was also higher in cells maintaining a rapid outgrowth of neurites when compared with cells with identical past histories but with intact, fully developed neurites. This last result provided further evidence to the previous hypothesis (Deanin et al., 1981) that the turnover of carboxyterminal tyrosine is more closely correlated with rapid changes in the cell shape than with the maintenance of cellular asymmetries.

Tyrosination State of Tubulin and Microtubules

Tyrosination state refers to the proportion of tubulin molecules that contain a tyrosine residue at the carboxyl terminus of its α-chain in a specific pool. As has already been mentioned, phenylalanine is also found at the carboxyl end of α-tubulin (Barra et al., 1973b; Rodríguez et al., 1975; Arce et al., 1975). However, since the relative amounts of tubulin containing phenylalanine is very low in comparison with tyrosine (Rodríguez and Borisy, 1978), we obviated, for simplicity, this minor species of tubulin.

The first studies on the amino acid sequence of the carboxy-terminal region of α-tubulin indicated the presence of about 0.3 and 0.15 mol of tyrosine/mol of tubulin isolated from brain of beef (Lu and Elzinga, 1978) and pig (Ponstingl et al., 1979), respectively. By using a method based on the [^{14}C]tyrosine acceptor capacities of tubulin before and after treatment with pancreatic carboxypeptidase A, Raybin and Flavin (1977b) showed that bovine brain tubulin purified by 3 cycles of assembly-disassembly contained about 12 and 25% of tyrosinated and nontyrosinated tubulin, respectively. Even if a non-substrate tubulin (*see below*) could account for the remainder, those values probably do not reflect the actual degree of tyrosination of tubulin in the brain tissue since no correction was done for the amount of tyrosine that may have been released during the warm incubations carried out to isolate tubulin (Arce and Barra, 1985).

Rodríguez and Borisy (1978) measured the amount of tyrosine released by the action of pancreatic carboxypeptidase A on purified chick brain tubulin and showed that nearly 50% of tubulin was tyrosinated during embryonic stages. This value decayed to 32% in the adult animal. Detyrosinated tubulin, as measured by its tyrosine-acceptor capacity, reached up to 27% in the embryo and fell to 12% in the adult. A third species of tubulin, which is a non-substrate for the ligase, became apparent. This non-tyrosinable tubulin was present in low amounts immediately before hatching (23%) and increased up to 55% in the adult animal.

Nath and Flavin (1979) measured the tyrosine-acceptor capacity of tubulin and found that in neuroblastom-glioma hybrid cells, the tyrosination state of tubulin changed after cAMP-induced differentiation. The amount of tyrosinated and detyrosinated tubulin was also measured in squid optic ganglion and in flagella of sea urchin sperm (Kobayashi and Matsumoto, 1982).

It is important to point out that the determinations of tyrosine-acceptor capacities of tubulin mentioned above were carried out without corrections for inactivation of the tyrosination system. We determined the tyrosination state of rat brain tubulin during development controlling rigorously the possible inactivation of the tyrosination system during the isolation, pretreatment with carboxypeptidase A, and [^{14}C] tyrosine incorporation (Barra et al., 1980). In addition, to avoid the problems inherent to the colchicine-binding method, we measured total soluble tubulin by a method based on the relationship of specific radioactivity between [^{14}C] tyrosinated tubulin purified from brain extracts and that measured in the starting material (Barra et al., 1980). We found that the amount of tyrosinated and detyrosinated tubulin species decreased progressively with the development of the animal. The non-substrate species was in low amount (15%) in the 4-d-old rats and increased up to 48% in the adult. Soluble tubulin from rat brain synaptosomes was shown to be composed by tyrosinated and detyrosinated species in a ratio similar to that found in total brain (Barra and Arce, 1983).

What is the distribution of tyrosinated and detyrosinated tubulin between microtubules and the nonassembled tubulin pool? Rodríguez and Borisy (1979a) reported that in brain tissue from newborn rats the proportion of tyrosinated tubulin in the nonassembled fraction is higher than that in the microtubules. By using

antibodies specific to tyrosinated and detyrosinated tubulin an important contribution on this question has emerged from Bulinski's laboratory. In cultured monkey kidney cells it was found that some microtubules stained well with the antibody to tyrosinated tubulin and some stained well with the antibody to detyrosinated tubulin (Gundersen et al., 1984). In addition, some microtubules were stained with both antibodies. Cytoplasmic detyrosinated microtubules were more limited in number and length than tyrosinated microtubules and were characterized as having a curly morphology. Similar results have also been found in mitotic spindles in this and other cell lines (Gundersen and Bulinski, 1986a). In proliferating PtK1 cells, tyrosinated tubulin was the predominant form in almost every cytoplasmic microtubule (Gundersen and Bulinski, 1986b). In contrast, staining of centrioles and primary cilia of PtK1 cells suggested that detyrosinated tubulin was the predominant form in these stable assemblies of microtubules. Stable microtubules present in cultured neuronal cells (neurites), sperm and tracheal cells (axonemes and basal bodies), and platelets and erythrocytes (marginal bands) contained more detyrosinated tubulin than interphase or spindle microtubules of proliferating cells (Gundersen and Bulinski, 1986b). The one exception, the marginal band of toad erythrocytes, which contained only tyrosinated tubulin, could indicate that an elevated level of detyrosinated tubulin is not an obligate feature of a stable array of microtubules. Since detyrosinated microtubules arise from tyrosinated microtubules (Gundersen et al., 1987), it is possible that toad erythrocytes do not express tubulin carboxypeptidase. Differences in the distribution of the two tubulin species were confirmed by electron microscopic analysis using immunogold staining of microtubules (Geuens et al., 1986).

The question on the relationship between microtubule stability and the degree of tyrosination was examined recently by Kreis (1987) by immunofluorescent techniques after injection of labeled tubulin into Vero cells. He showed that microtubules rich in tyrosinated tubulin are very dynamic structures, whereas those rich in detyrosinated tubulin are more stable.

The tyrosination state of microtubules by immunocytochemical techniques has also been investigated in nervous tissues. It was found that in rat cerebellum, microtubules of the parallel fiber axons become more detyrosinated with the age of the animal (Cumming et al., 1984). This could be explained by the progressive action of tubulin carboxypeptidase on microtubules during maturation of axons. Another immunocytochemical study performed in axons of spinal motor neurons suggests that although microtubules contain a low amount of tyrosinated tubulin, the detyrosination process does not occur during the transport of tubulin from the cell body (Burgoyne and Norman, 1986). Therefore, detyrosination would take place in or near the cell body or on microtubules rather than transported monomer.

As was mentioned, α-tubulin can also be modified by acetylation of lysine residues. A monoclonal antibody specific for acetylated α-tubulin has recently been developed (Piperno and Fuller, 1985). By using this antibody, the distribution acetylated α-tubulin in rat cerebellum was examined and compared with that of total α-tubulin and detyrosinated α-tubulin (Cambray-Deakin and Burgoyne, 1987). According to this study acetylated α-tubulin appears to be codistributed with detyrosinated α-tubulin, both forms being predominantly present in parallel fiber axons in adult cerebellum. It would be of interest to know the relationship between microtubules with low rate of turnover and microtubules containing acetylated and detyrosinated α-tubulin. It is possible that acetylation confers stability to the microtubules, and consequently, microtubules would be exposed for a longer time to the action of the carboxypeptidase producing more detyrosinated microtubules.

Tyrosination State in Relation to Physiological Roles of Tubulin and/or Microtubules

After the discovery of tubulin tyrosination, the possible influence of the tyrosination state of tubulin on the formation of microtubules was rapidly investigated. In vitro experiments demonstrated that whatever the tyrosination state of tubulin, microtubules could be formed with the same efficiency (Arce et al., 1978; Kumar and Flavin, 1982a). The polymerization-competence of these tubulin species was also demonstrated to occur in vivo (Gundersen et al., 1987; Webster et al., 1987). Tubulin containing phenylalanine instead of tyrosine at the carboxyl end behaved similarly to tyrosinated or detyrosinated tubulin (Arce et al., 1978). It should be mentioned that in the brain of newborn rats the proportion of tubulin molecules containing carboxyterminal phenylalanine, which represents 4% of the aminoacylated tubulin, increases up to eightfold after induction of hyperphenylalaninemia and the pool of tyrosine-containing molecules decreases by an equivalent amount (Rodríguez and Borisy, 1979b). The chemical difference between tyrosine and phenylalanine could affect the capacity of microtubules to interact with other cellular elements. Nevertheless, whether or not the pathogenesis of brain dysfunction in phenylketonuric individuals is related to this modification needs to be investigated.

Theoretical analysis of tubulin structure suggests that the presence or the absence of a tyrosine residue at the carboxyterminal position of a-tubulin is able to modify the secondary structure of the carboxyterminal region (Ponstingl et al., 1979). Moreover, it has been shown that the acidic carboxyterminal regions of α- and β-tubulin can modulate the assembly properties of the protein (Serrano et al., 1984a,b). Thus, even though the tyrosination of the carboxyl terminus of α-tubulin does not seem to affect the assembly capacity of tubulin in vitro, it is possible that this modification influences in some way the interaction of tubulin with other macromolecules.

Some subtle differences in the behavior of tyrosinated and detyrosinated tubulin were described by Kumar and Flavin (1982a). A smaller proportion of MAPs were detected on microtubules formed from detyrosinated tubulin. In addition, during taxol-induced assembly, the rate and extent of the process was about twofold higher with tyrosinated tubulin.

Although investigators agree with the idea that the tyrosination state does not alter microtubule assembly in vitro, the hypothesis that it might be regulating some function of microtubules in vivo has in no way been discarded. In this respect the work by Matsumoto et al. (1983), can be mentioned, which found that tyrosinated tubulin is necessary for maintenance of membrane excitability in squid giant axon. Microtubules, at least those underlying the axolemma, should contain tyrosinated tubulin to produce, by an unknown mechanism, favorable conditions for membrane excitability.

The fact that tubulin is a suitable substrate for insulin receptor kinase (Kodowaki et al., 1985), is remarkable. When the substrate was tyrosinated tubulin, phosphate was incorporated on the carboxyl-terminal tyrosine and the phosphorylated protein did not assemble into polymers (Wandosell et al., 1987). Whether this modification occurs in vivo and regulates tubulin polymerization remains to be determined.

A number of studies have indicated the presence of tubulin in isolated membrane fractions (Feit and Barondes, 1970; Bhattacharyya and Wolf, 1975). However, the molecular or functional basis of the tubulin-membrane interaction remains to be elucidated. We found that purified tubulin associates with microsomal membranes from rat brain and that this association is independent of the state of tyrosination of tubulin (Rodríguez and Barra, 1983).

There are other works in which correlations between the tyrosination state of tubulin

and some physiological states have been investigated. Investigation on the tyrosination state in oocytes from *Bufo arenarum* revealed that soluble tubulin is composed mainly (95%) by the non-substrate species (Barra et al., 1987). A low proportion (4–5%) of tyrosinated tubulin was also detected, but practically none of the detyrosinated tubulin species. This pattern did not change after fertilization and during early embryogenesis (up to 22 h post-fertilization). On the other hand, studies on tubulin from *Xenopus laevis* oocytes (Preston et al., 1981) showed that detyrosinated tubulin is present at stage 5/6 oocytes and increases 3.5-fold after fertilization, whereas detyrosinated tubulin was not detected at stages 2–4.

The studies on the tyrosination state described above were carried out after isolation of total soluble tubulin, that is, without distinguishing those molecules constituent of the assembled and the nonassembled pools. Rodríguez and Borisy (1979a) showed that the tyrosination state of both pools changed with development. In newborn rats, the content of tyrosinated species in the nonassembled pool was twice as high as that in microtubules. During development tyrosinated tubulin decreased and became practically equal to the amount in the assembled pool.

In conclusion, the report of Rodríguez and Borisy (1979a) indicated that, at least in early stages of development, tyrosinated and detyrosinated tubulin do not distribute at random between the two states of assembly.

Bulinski et al. (1980) found no difference in the tyrosination state of tubulin isolated from exponentially growing and mitotic cultured HeLa cells. More recently, Gundersen and Bulinski (1986a) investigated the distribution of tyrosinated and detyrosinated tubulin during mitosis of cultured cells by immunofluorescence using antibodies that specifically react with each of the forms. The distribution of tyrosinated tubulin differed from that of detyrosinated tubulin at each stage of mitosis. In general, the distribution of tyrosinated tubulin was similar to that of total tubulin, whereas detyrosinated tubulin had a more restricted distribution. Tyrosinated tubulin is the major species in the mitotic spindle and is found in all classes of spindle fibers, whereas detyrosinated tubulin is present in small amounts at restricted areas.

Recently, a new approach to investigate the significance of the tyrosination of tubulin has been developed by Wehland et al. (1986). They obtained a polyclonal antibody against tubulin: tyrosine ligase and showed that the injection of this antibody to swiss 3T3 cells leads to the nonreactivity of the YL 1/2 antibody with the microtubular network. This lack of tyrosinated microtubules suggests that the incorporation of tyrosine into the carboxyl terminus of a-tubulin in vivo was efficiently inhibited. By inhibition of the ligase in this way and later analysis of the microtubules with antibodies against tyrosinated and detyrosinated tubulin, Wehland and Weber (1987b) studied some aspects of the mechanism of detyrosination of a-tubulin. They found that in certain permanent cell line, such as 3T3, tubulin carboxypeptidase acts slowly throughout the cell cycle. On the other hand, in other cell lines such as CHO, or in cells with the potential to differentiate, the carboxypeptidase practically does not act as the cells proliferate. When differentiation was initiated by the addition of forskolin (an activator of adenylate cyclase), elevated levels of detyrosinated microtubules were observed, suggesting an induction or activation or the carboxypeptidase through cyclic AMP-dependent events.

In another type of experiment the monoclonal antibody YL 1/2 was microinjected into Swiss 3T3 fibroblasts (Wehland et al., 1983). Low concentrations of the antibody did not alter the normal arrangement of microtubules, whereas higher concentrations induced the formation of bundles that aggregated around the nucleus. Furthermore, depending on the concentration, YL 1/2 abolished saltatory movements, cell translocation, and mitosis, and

caused dispersion of the Golgi complex (Wehland and Willingham, 1983).

Cycle of the Tyrosination/ Detyrosination Reaction

The overall reaction (tyrosination/detyrosination) comprising the two main states of aggregation of tubulin is represented in Fig. 2.

The principal findings in relation to this cycle can be summarized as follows:

A) The formation of microtubules would not depend on the state of tyrosination, since tyrosinated and detyrosinated tubulin assemble equally well in vitro. The carboxyterminal tyrosine can be phosphorylated by insulin receptor kinase. This modification prevents tubulin polymerization in vitro.

B) The ligase can act reversibly. However, under all conditions studied the net direction is toward the formation of tyrosinated tubulin. The incorporation reaction requires ATP, Mg^{2+}, and K^+, whereas the reverse reaction requires ADP, Pi, Mg^{2+}, and K^+. At present, no other physiological activator or inhibitor of this enzyme has been described. In vitro, the enzyme incorporates tyrosine only on nonassembled tubulin. In vivo, it uses nonassembled tubulin, but we cannot rule out the possibility that the enzyme uses also polymeric tubulin as a substrate.

C) Polymeric tubulin is a better substrate than nonassembled tubulin for tubulin carboxypeptidase. When microtubules and tubulin are in equilibrium, detyrosination occurs exclusively on microtubules. In vivo, like in vitro, the enzyme seems to use polymeric tubulin.

D) Several natural compounds were shown to activate or inhibit the carboxypeptidase activity. The enzyme activity can be stimulated by the polycations spermine, spermidine, and putrescine. The enzyme can be inhibited by soluble RNA, proteoglycans, and basic proteins present in soluble brain extracts. In addition, several synthetic polyanions and polycations also inhibited the detyrosination reaction, and among those tested heparin was the most effective.

According to the data presented above, the state of tyrosination of microtubules should depend on: (a) the state of tyrosination of tubulin to be assembled, (b) the turnover of microtubules, and (c) the activity of carboxypeptidase (assuming that ligase does not use polymeric tubulin as a substrate). Considering the preference of the ligase and carboxypeptidase for tubulin and microtubules, respectively, the ratio of tyrosinated to detyrosinated tubulin in vivo is expected to be higher in the nonassembled tubulin pool than in microtubules. This difference depends not only on the relative activities of these enzymes but also on the rate of the turnover of microtubules, that is, the exchange of tubulin between microtubules and nonassembled tubulin. If the exchange is slow, microtubules will become more detyrosinated over time, whereas nonassembled tubulin will tend to be completely tyrosinated. On the other hand, if the exchange is very rapid the breakdown of microtubules will yield little detyrosinated tubulin, since little detyrosination will have occurred during the short life of the polymers. Consequently, the nonassembled tubulin will be maintained in a highly tyrosinated state and the difference in the tyrosination state between tubulin and microtubules will tend to disappear owing to the mixing of the pools.

According to the model presented, whatever the rate of exchange, the nonassembled tubulin is expected to be almost completely tyrosinated. However, Rodríguez and Borisy (1979a) did detect a significant amount of detyrosinated tubulin in the nonassembled fraction prepared from brain. This monomeric detyrosinated tubulin could arise from: (1) inefficiency of the ligase in retyrosinating tubulin, (2) expression of α-tubulin (*see* the Genetic Variability section), or (3) breakdown of native microtubules during the homogenization procedure.

As mentioned above, the activities of ligase and carboxypeptidase depend on several effec-

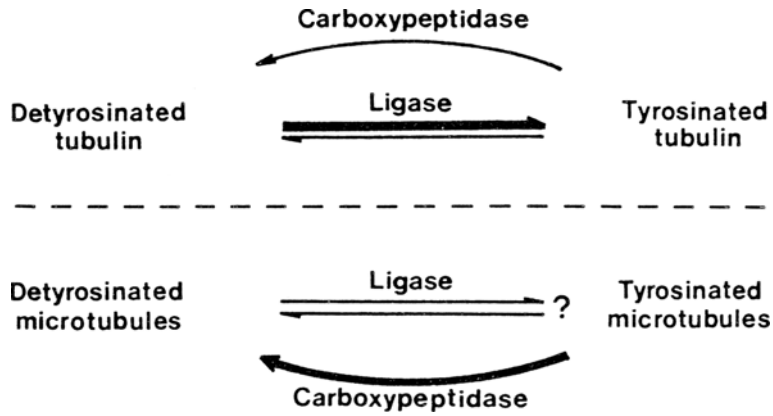

Fig. 2. Overall tyrosination/detyrosination reaction of tubulin and microtubules. The dashed line represents the separation between both states of aggregation, microtubules, and free tubulin dimers.

tors. Although the action of activators and inhibitors has not been studied in vivo some of these may be compartmentalized, producing microtubules with variables states of tyrosination according to the function in which they are involved.

Genetic Variability of Tubulin

In earlier stages of the investigation on the cyclic tyrosination/detyrosination of α-tubulin, the tyrosination reaction was assumed to occur before detyrosination. In 1981, Valenzuela et al., (1981) reported the first nucleotide sequence of an α- and β-tubulin gene; the α-tubulin amino acid sequence deduced showed a tyrosine residue immediately before the stop codon. From this result it was concluded that the primary posttranslational modification of the carboxyl terminus of α-tubulin was the release and not the addition of the tyrosine residue. This possibility was challenged by the later finding of an α-tubulin gene without carboxyterminal tyrosine encoded (Villasante et al., 1986).

In the last years a great amount of information has been rapidly accumulated with respect to DNAs sequences of the multigene family that encodes α- and β-tubulin. It is not the purpose of this section to review all the available data about the genetics of tubulin but to analyze how the genetic variability of tubulin could be related to some aspect concerning the reversible posttranslational modification of the carboxyl terminus of α-tubulin.

Extensive sequence analyses of the tubulin gene family in mouse and chicken have derived principally from Cleveland's and Cowan's laboratories. In mouse, six functional α-genes encode five distinct α-tubulin isotypes, and five β-genes encode five different β-tubulin isotypes (Lewis et al., 1985; Villasante et al., 1986; Wang et al., 1986). One of the most divergent regions among the α-isotypes and among the β-isotypes is the carboxyterminal 15 residues. Among the α-isotypes, five have encoded a carboxyterminal tyrosine before the stop codon and one has glutamic acid in that position.

Therefore, the question is, which of these different isotype species is able to participate in the tyrosination/detyrosination reaction? Is it tyrosinated or detyrosinated?

In mouse nervous tissue, three α-tubulin genes are expressed: two of these, having encoded the carboxyterminal tyrosine, are expressed mainly in early stages of development. The other gene, which has no tyrosine but glutamic acid encoded as the carboxyterminal amino acid, is expressed predominantly in the adult animal (Villasante et al., 1986). Relating these

boxyterminal tyrosine, it is obvious that after tubulin synthesis, detyrosination should occur before tyrosination. Second, in adult rat brain extracts about 50% of tubulin is not able to accept tyrosine under any known condition (Barra et al., 1980) and apparently this non-substrate tubulin does not contain carboxyterminal tyrosine (Rodríguez and Borisy, 1979a). Therefore, it is possible that this nonacceptor tubulin is the product of the expression of the α-tubulin gene that does not have carboxyterminal tyrosine encoded since, in addition, it was shown to be highly divergent (Villasante et al., 1986).

Other explanations for the presence of this non-substrate tubulin fraction can be considered in addition to that mentioned above. One takes into account recent results obtained by Wehland and Weber (1987) who showed that tubulin:tyrosine ligase has its binding site on β-tubulin. It is possible to speculate that the product of a divergent β-tubulin gene was not able to bind the ligase, thus giving rise to a tubulin dimer that cannot accept tyrosine. Another explanation is based on the possibility that another posttranslational modification of the tubulin molecule takes place producing a non-tyrosinable tubulin species.

In *Drosophila melanogaster* four α-tubulin genes have been sequenced; three of them show a tyrosine codon before the stop codon, whereas in the fourth one a phenylalanine residue is encoded instead of tyrosine (Theurkauf et al., 1986).

The analysis of the genes of α-tubulin, particularly at the region corresponding to the carboxyl terminus of the polypeptide, became more complex from the work by Pratt et al. (1987), who reported the presence of a serine residue encoded before the stop codon in a gene that is expressed exclusively in chicken testes. The functional role of this α-isotype is at present unknown.

In lower eukaryotes genetic divergence between different tubulin genes has been also detected. In *Chlamydomonas reinhardtii*, the DNA sequence of two α-tubulin genes indicated the presence of an encoded carboxyterminal tyrosine in both genes (Silflow et al., 1985). In *Saccharomyces cerevisiae*, two α-tubulin genes codify for a phenylalanine in the carboxyterminal position of the protein (Yanagida, 1987). In *Schizosaccharomyces pombe*, two α-genes have been sequenced, both showing a tyrosine codon before the stop codon (Yanagida, 1987). In *Physarum polycephalum*, two different α-tubulin genes have been sequenced. One of the genes has a methionine carboxyterminal residue encoded (Monteiro and Cox, 1987), whereas the other gene shows a deletion of 26 codons corresponding to the carboxyl terminus of the protein (Krammer et al., 1985). Although detailed analysis employing tubulin mutants and cloned genes have revealed different cellular roles for the tubulin genes in these organisms (Yanagida, 1987) no attempt has been made to investigate the possible role of the aromatic carboxyterminal residue in lower eukaryotes.

Perspectives

With the contribution of recent biochemical, immunological, and genetic studies we have a more detailed description of the enzymatic reactions that lead to the tyrosination/detyrosination of tubulin. The studies performed up to this point have provided some insight into the system but have also generated many questions Which of the different tubulin isotypes is the substrate of this reaction? Do tyrosinable and non-tyrosinable tubulin have different functions? Is tubulin carboxypeptidase in the cell associated with microtubules? If tubulin carboxypeptidase is associated with microtubules, is its distribution homogeneous? What is the relationship between acetylated α-tubulin and detyrosinated microtubules? Detyrosinated microtubules are more stable than tyrosinated microtubules. Is this posttranslational modifi-

cation the cause or the consequence of microtubule stability? What is the influence of the endogenous inhibitors and activators of tubulin carboxypeptidase on the tyrosination state of microtubules? Is the occurrence of microtubules with different levels of tyrosination regulated by the activity and distribution of these effectors? Although it is possible that the tyrosination state of microtubules modifies the interaction with other cellular components and regulates some function carried out by these structures, much work remains to be done to have some insight about the physiological role of these reactions.

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