TYROSYLTUBULIN LIGASE AND COLCHICINE BINDING ACTIVITY IN SYNCHRONIZED CHINESE HAMSTER CELLS

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ABSTRACT

Tyrosyltubulin ligase (TTL) was found to be present in CHO and V79 Chinese hamster cells grown in tissue culture. The enzyme is soluble and requires potassium, magnesium, and ATP for maximum activity and requires tubulin as a substrate.

TTL was analyzed through the cell cycle of V79 and CHO Chinese hamster cells. The enzyme showed two peaks of activity in V79 cells at 4 h and 7 h after mitotic selection, corresponding to the early S and mid to late S phases of the cell cycle. In CHO cells the enzyme displayed a major peak of activity at mid S and a minor peak or plateau during early S. Tubulin, as measured by [^{3}H]colchicine binding, was shown to increase through S phase and reach a maximum late in the cycle during G2 approx. 3 h after maximum TTL activity.

KEY WORDS tyrosyltubulin ligase · tubulin · cell cycle · colchicine binding · chinese hamster · tissue culture

Tubulin, the major subunit of microtubules, is among the most ubiquitous of proteins. It is a major component of the mitotic apparatus and therefore has an implicit importance in the control of cell division especially in rapidly dividing cells. In addition to its role in cell division, it plays a major role in many other cellular functions such as motility, secretion, transport and in the maintenance of morphology (6, 7, 11, 15, 16, 21, 22, 30, 36, 37). A role for microtubules has also been postulated for the organization of the cell surface (1, 28, 38), and Brinkley et al. (8) have shown important differences in microtubular structures between normal and transformed cells. The role of microtubules in living cells appears so diverse in function that a common mode of regulation for all functions may not exist. There have been several possible mechanisms suggested by which the function of tubulin could be regulated; these include the phosphorylation of serine residues in β-tubulin (12, 14), the binding of GTF to specific sites on tubulin (40), the inhibitory effects of Ca^{++} and the requirement for Mg^{++} in tubulin polymerization (29, 39), and the stimulation of polymerization by the microtubule-associated proteins (26).

In addition to the above interactions, Barra et al. (3) and Raybin and Flavin (31) have described an enzyme, tyrosyltubulin ligase (TTL), which adds tyrosine to the carboxyl terminal glutamine or glutamate of α-tubulin. TTL is found in high concentrations in the brain and in other neural tissues where large quantities of tubulin are also found. In the present report, we describe the presence of TTL in tissue culture cells and assay its pattern of activity through the cell cycle of two Chinese hamster cell lines in which the pattern of tubulin synthesis has been described previously (13).

MATERIALS AND METHODS

Cell Culture and Synchrony

Cells were grown in *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffered McCoy's 5a modified medium with 5% or 10% fetal calf serum (FCS). Mitotic cells were automatically selected and pumped into flasks with a Talandic cell cycle analyzer (model 2001; Talandic Research Corp., Pasadena, Calif.) as previously described (18). Suspension culture cells were synchronized by suspending cells in isoleucine-free medium for 20 h (25) and then pumping the cells into an equal volume of fresh medium containing 2 × the normal isoleucine concentration at hourly intervals, with a model 2004 Talandic cell cycle analyzer. Synchrony was monitored with an Hitachi time-lapse video tape recorder. DNA synthesis was determined by incubating the cells with [^{3}H]thymidine as reported previously (13).

TTL Assay

Monolayer cultures were harvested by a brief trypsin treatment, centrifuged, and resuspended in 10 ml of Hanks' balanced salt solution (BSS). The cells were counted and centrifuged 2,000 *g* for 2 min. The cell pellet was resuspended in TTL buffer (0.05 M Tris pH 7.4, 0.015 M Mg acetate, 0.1 M KCl, 1 mM dithiothreitol (DTT), 1 M glycerol, 200 μg/ml RNase) at a concn of approx. 4×10^6 cells/ml and sonicated with a Bronwill sonicator (Bronwill Scientific, Rochester, N. Y.) for 10 s with the probe intensity setting at 80. Cells grown in suspension culture were counted directly with a model A Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.), centrifuged, and the cell pellet was resuspended in TTL buffer and sonicated. The sonicated cells were centrifuged at 224,000 *g* max for 1 h, and the supernate and pellet fractions were assayed for TTL activity. The assay mixture was incubated at 37°C for various times up to 2.5 h and contained the following reagents in addition to the TTL buffer: 4 mM ATP, 2.25×10^{-8} M [^{3}H]tyrosine (48–51 Ci/mmol, New England Nuclear, Boston, Mass.) and 200 μg/ml purified rat tubulin. Final reaction volume was 200 μl. The rat tubulin was purified by two cycles in an in vitro polymerization system described by Shelanski et al. (35). The assay was stopped by placing the samples in ice and immediately pipetting two 100-μl samples into 1 ml of 10% trichloroacetic acid (TCA) containing 20 μg of unlabeled tyrosine. BSA (200 μg) was added as carrier, and the TCA precipitates were kept at 0°C for 15–30 min. The TCA precipitates were collected on 0.45-μm Millipore filters (Millipore Corp., Bedford, Mass.) or on GFC Whatman glass fiber filters. The samples were washed three times with 5 ml of 10% TCA, dried, dissolved in 10 ml of Aquasol (New England Nuclear, Boston, Mass.) and counted in a Beckman scintillation counter (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.).

Colchicine Binding

Colchicine binding was assayed on sonicated cell lysates in 0.01 M phosphate buffer pH 6.8 containing 0.1 M sodium glutamate. [^{3}H]colchicine (2×10^{-6} M final concentration) was added, and the samples were incubated at 37°C for 2 h. The samples were filtered through DE81 filters and assayed according to the procedure of Borisy (5). Blanks for each sample were run as described above but contained in addition a 1,000-fold excess of unlabeled colchicine.

Polyacrylamide Gel Electrophoresis

The α- and β-subunits of tubulin were separated by electrophoresis in 6 M urea 10% polyacrylamide gels. The system of Laemmli (23) was used except that urea was added to a final concentration of 6 M and the buffer in the running gel and spacer gel was at one-half the ionic strength of that used by Laemmli. The proteins were stained with Coomassie Blue and photographed to show the α- and β-tubulin subunits. The gel was impregnated with the scintillator 2,5-diphenyloxazole (PPO) and prepared for fluorography according to the procedure of Laskey and Mills (24). Kodak RP Royal X-Omat film was used to detect the tyrosylated protein bands.

RESULTS

Characteristics of CHO TTL

Table I presents some of the characteristics of the TTL enzyme found in CHO cells. The CHO-TTL enzyme can use purified rat brain tubulin as a substrate. The enzyme requires magnesium, potassium, and ATP for full activity. These are

TABLE I

Characteristics of the CHO Tyrosyltubulin Ligase Enzyme

Sample	[^{3}H]Tyrosine incorporated
	%
Complete system	100*
Minus ATP	5.4
Minus Mg^{2+}	<1
Minus K^+	6
Plus unlabeled tyrosine (1.7 mM final)	<1
Plus unlabeled phenylalanine (2.4 mM final)	32
Minus rat tubulin substrate	<1
Minus enzyme sample (blank) (activity present in rat tubulin substrate)	3.1
Complete system on ice	<1

Cell supernate represents 4×10^6 cells/ml sonicate solution.

* 100% = 4,632 cpm/assay.

the same requirements that have been described for the enzyme isolated from rat brain (2, 4). Incorporation of [^{3}H]tyrosine was inhibited when unlabeled tyrosine or phenylalanine was added. Unlabeled tyrosine at 1.7 mM completely inhibited incorporation while phenylalanine at 2.5 mM inhibited tyrosine incorporation by 68%. The dependence upon added rat tubulin for tyrosine incorporation is shown in Table I. Absence of added rat tubulin as substrate in the assay system resulted in no [^{3}H]tyrosine incorporation. One might have expected tyrosine to be incorporated into the endogenous tubulin of the CHO cells. However, the absence of incorporation suggests that insufficient amounts of endogenous tubulin are present or that the CHO tubulin is already tyrosylated. In addition, when we tried to use either the particulate material or the supernate as a tubulin source, there was no detectable [^{3}H]tyrosine incorporated (not shown). Incubation of an assay mixture without enzyme resulted in a small amount (3.1%) of [^{3}H]tyrosine incorporation. This was due to residual enzyme activity in the purified rat tubulin used as a substrate and was corrected by running a blank with each experiment (Table I, row 8).

Tyrosylation by the CHO-TTL enzyme is specific for the α-subunits of tubulin as is shown in Fig. 1. The gel on the left in Fig. 1 shows the separation of the α- and β-tubulin subunits stained with Coomassie Blue while the right portion of Fig. 1 shows a fluorograph of tubulin tyrosylated with [^{14}C]tyrosine with the CHO-TTL enzyme. It is clear that specificity is for the α-subunit. The specificity of brain TTL for the α-subunit has been described previously (31). The specific activity of TTL was determined in the 100,000 *g* supernate of CHO exponential phase cell lysates. Tubulin substrate concentration was 500 μg/ml, and [^{14}C]tyrosine was at 4 μM final concentration. The specific activity was measured at 2.3 pmol/min/mg which is in the range of published values (2–120 pmol/min/mg) (Raybin and Flavin [32]; Deanin and Gordon [10]).

Table II reviews the optimization of conditions used during processing of the CHO extracts that might affect the activity or stability of the enzyme. Raybin and Flavin (31) reported that the particulate material from rat brain had to be removed from the reaction mixture before assay or inactivation of the enzyme would result, presumably through adsorption of the enzyme to the particulate material. They have recently shown that TTL binds to tubulin which is found in large quantities in the particulate material of brain (33). This binding and inactivation does not take place in CHO cells as shown in Table II. Almost all of the

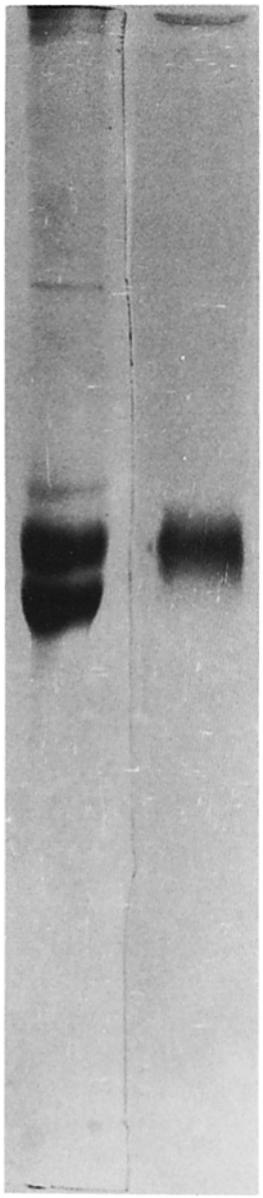

FIGURE 1 Tyrosylation of the tubulin α-chains. TTL from CHO cells was used to label rat tubulin with [^{14}C]tyrosine (see Results for details). The left column shows the Coomassie Blue-staining pattern for the α- and β-subunits. The right column is a fluorogram of the [^{14}C]tyrosine-labeled rat tubulin showing specific labeling of the α-subunits. Both samples were analyzed in adjacent slots in polyacrylamide slab gels.

TABLE II

Characteristics of the CHO Tyrosyltubulin Ligase Assay System

Sample	[^{3}H]Tyrosine incorporated
	%
CHO sonicate solution (particulate and soluble)	100*
CHO 224,000 *g* max supernate	93.7–100
CHO 224,000 *g* max pellet	7.5–0
Cell sonicate solution minus rat tubulin substrate	3
Sonication 5 s	100
Sonication 20 s	105
Conditioned medium	<1
Fresh medium	1.7
Activity after 24 h @ −80°C	106
Activity after 3 h @ 0°C	108

* 100% = 4,632 cpm/assay.

activity was located in the supernatant solution, with less than 10% in the pellet. These results were repeated several times with the supernate enzyme activity varying from 93.7% to 100% of the total TTL activity, with the pellet giving the complementary difference. The total cell lysate was incubated without substrate to show that the particulate material does not contain specific or nonspecific tyrosine-binding sites. The amount of tyrosine incorporated was negligible at 3%. We have already shown in Table I, row 7, that the 100,000 *g* supernate solution which contained >90% of the endogenous tubulin gave no detectable tyrosine incorporation when rat tubulin substrate was deleted. The enzyme was stable to sonication for 5–20 s.

Tissue culture medium supplemented with FCS is often a source of enzyme contamination. Table II shows that our culture medium had an insignificant amount of TTL activity. In addition, conditioned medium did not contain any TTL activity, and thus there was no significant loss of active enzyme to the medium.

Barra et al. (4) reported that TTL from rat brain was very unstable at 0°–4°C (70% loss in activity in 4 h). Rodriguez et al. (34) subsequently showed that glycerol stabilized the enzyme with only a 20% loss of activity at 0°–4°C in 24 h. Table II shows that TTL activity in sonicated CHO cells was completely stable after 3 h at 0°C or after 24 h at −80°C in TTL buffer. In addition, the enzyme was stable in the intact cell at 0°C, losing only 6% of its activity in 1.5 h (data not shown). It takes approx. 30–45 min to process the cells through the sonication step, and therefore our maximum loss of enzyme activity was <6%.

In Fig. 2, we have determined some of the conditions which are necessary for optimal measurement of TTL through the cell cycle. Fig. 2*a* and *b* depict two separate experiments showing that the enzyme reaction is linear from 10 min to 2.5 h. The number of cells analyzed in Fig. 2*b* was 5 × the number analyzed in Fig. 2*a*. Some differences in tyrosine incorporation were noted when different tubulin preparations and different lots of [^{3}H]tyrosine were used. These differences were eliminated by using the same lots of tubulin and [^{3}H]tyrosine in comparable experiments. In Fig. 2*c*, we have determined the number of cells in an exponentially growing culture necessary to give a near linear reaction during a 2.5-h assay. The reaction was directly proportional to cell number from 3.4×10^5 to 1.7×10^6 cells/ml of assay mixture. The following experiments were performed using cell numbers within the linear range shown in Fig. 2*c*.

The results in Fig. 2*c* also indicate that the exogenously added tyrosine is in sufficient excess over cellular tyrosine. The cell number increase of 3.5×10^5–1.7×10^6 represents a fivefold increase in cellular (unlabeled) tyrosine, but does not affect the rate of [^{3}H]tyrosine incorporation.

Cell Synchrony

Synchronous CHO and V79 cells were obtained by selecting mitotic cells from 750-cm^2 roller bottles and immediately plating them in 75-cm^2 plastic flasks. The degree of synchrony was monitored by video tape, and the results are shown in Fig. 3. The frequency of dividing cells was plotted against time. The synchronous CHO cells are shown in Fig. 3*a* and have a generation time of 12–14 h (19). Analysis of selected mitotic cells at the start of an experiment showed that >90% of the cells were in mitosis.

Fig. 3*b* shows the cell cycle times of V79 cells through two generations. These cells have an 8–9-h generation time and also display some heterogeneity after one cell cycle traverse (19).

Tyrosyltubulin Ligase Activity during the Cell Cycle

The role of TTL is unknown, but its specificity for tubulin naturally implies a role in regulation not only in brain but also in all tissues where it is found. We have analyzed its activity through the cell cycle of two Chinese hamster cell lines to

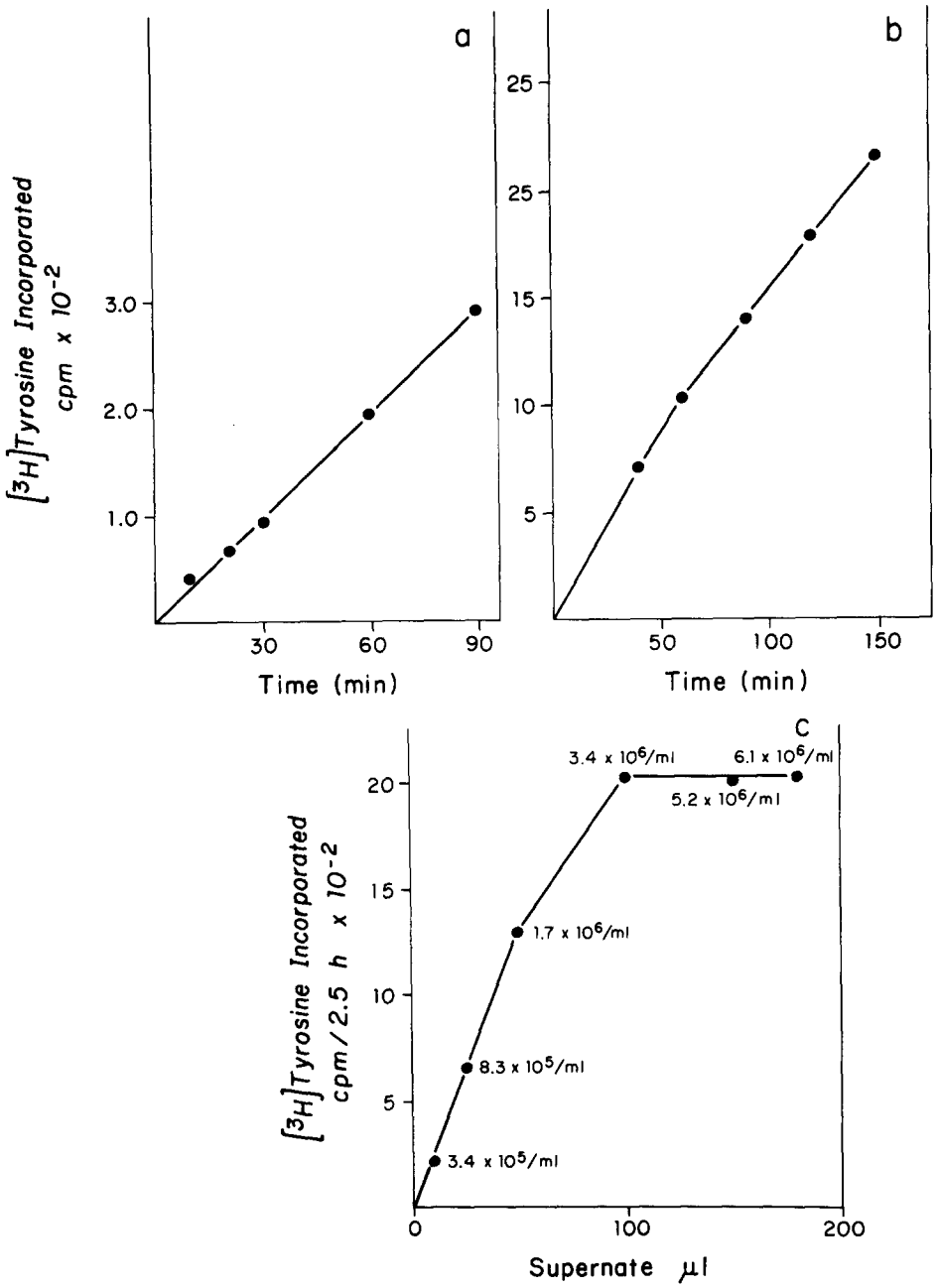

FIGURE 2 Relationship of TTL activity with time and with amount of cell extract. Fig. 2*a* and *b* show increasing TTL activity from a CHO 100,000 *g* supernate solution with time at 37°C. Fig. 2*b* contains five times more cells than Fig. 2*a*. Fig. 1*c* shows increasing TTL activity from a 100,000-*g* supernate solution with increasing enzyme. Final volumes were kept constant. The numbers by the data points represent cell numbers per milliliter of assay mixture. The reaction was incubated for 2.5 h at 37°C as described in Materials and Methods.

determine the pattern of activity and to establish its relationship to previously determined tubulin synthesis (13). Oscillations in enzyme activity can take place in short periods of time relative to the total cell cycle time in mammalian cells. Therefore we have taken 0.5-h samples when possible in order to better describe any fluctuations occurring during the cell cycle.

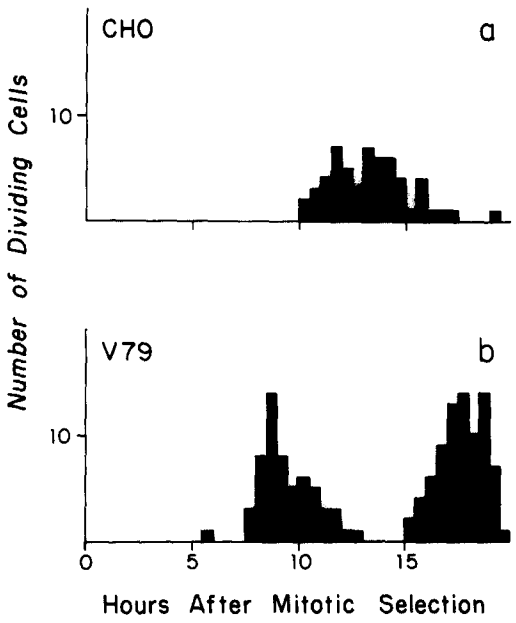

FIGURE 3 Histogram showing the frequency of cell division with time of mitotically selected V79 and CHO cells. Mitotic cells were selected and monitored by video tape at 37°C: (*a*) CHO cell cycle, and (*b*) V79 cell cycle.

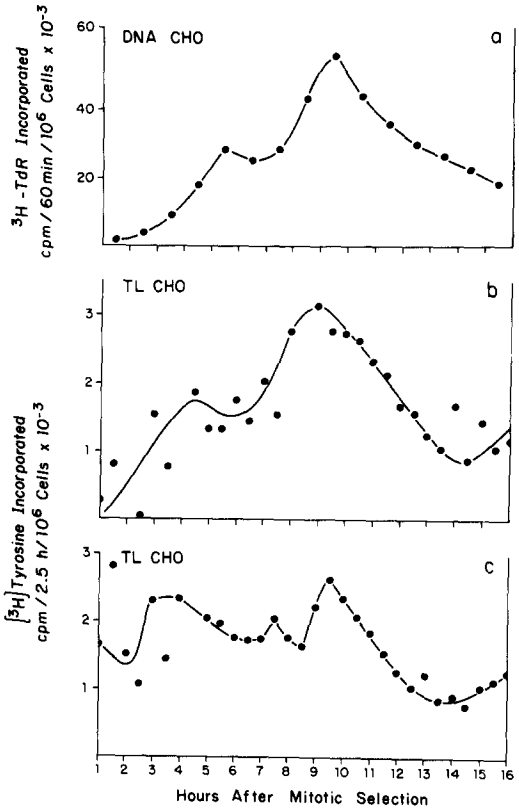

FIGURE 4 TTL activity and DNA synthesis during the CHO cell cycle. The method of synchrony was mitotic selection. (*a*) DNA in CHO cells was labeled for 1 h with 2 μCi/ml of ^{3}H-TdR at 1-h intervals throughout the cell cycle, and the acid precipitable counts were determined. (*b* and *c*) TTL activity in the CHO cell cycle from two synchrony experiments. Samples were taken every 30 min and assayed for 2.5 h at 37°C as described.

Fig. 4*b* and *c* show the cell cycle pattern of TTL in CHO cells. There is a major maximum in activity which peaks at 9–9.5 h into the cycle corresponding to the mid S phase of the CHO cell cycle. In addition to the major peak at 9–9.5 h, there was also a peak or plateau between 3.5 and 4.5 h in the cycle. This increase in activity occurred just before the first peak in DNA synthesis when the DNA synthetic rate was at a maximum. The Chinese hamster V79 line was also tested for cell cycle TTL activity. This line has a generation time 3–4 h shorter than that of CHO. The pattern for TTL activity in the V79 cell cycle is shown in Fig. 5*b*. Activity decreased during G1 and then increased sharply to a maximum during early S. A second major peak of activity was seen in mid S, 7 h into the cycle. The TTL activity then decreased during G2 to the same low point observed during G1. The TTL activity through the cell cycle in CHO and V79 gave patterns that were out of phase relative to the known patterns for tubulin synthesis (13). In order to show this phase relationship, CHO cells were synchronized in suspension culture by isoleucine starvation. This was done to provide enough cells to analyze for TTL activity and colchicine binding activity during the same experiment. After isoleucine reversal, samples were taken at hourly intervals for 24 h and analyzed for both TTL activity and colchicine-binding activity. The results are shown in Fig. 6. Colchicine-binding activity increased throughout S phase with maximum binding occurring in G2 just before cell division, while the TTL activity peaked in mid S 3 h before the maximum colchicine binding peak. It should be noted that the resolution from this synchronization procedure was not equal to that achieved by mitotic selection.

DISCUSSION

TTL is present in cultured Chinese hamster cells and appears to have the same characteristics as the enzyme originally found in rat brain (2, 4). It requires potassium, magnesium, and ATP for

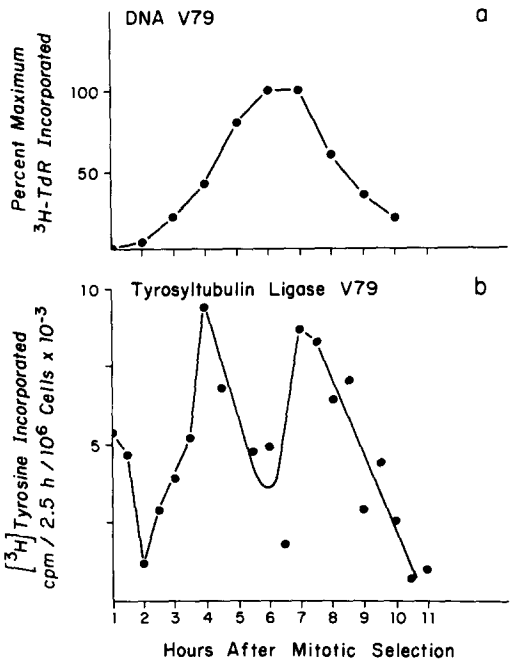

FIGURE 5 TTL activity and DNA synthesis in the V79 cell cycle. The method of synchrony was mitotic selection. (*a*) DNA in V79 cells was labeled for 30 min with ^{3}H-TdR, and the acid precipitable counts were determined. (*b*) TTL activity through the cell cycle in V79 cells. Samples were taken every 30 min and assayed for 2.5 h at 37°C.

maximum activity and uses tubulin as a substrate. Unlike most of the TTL in rat brain, this enzyme is not bound to the particulate fraction since greater than 93.7% of the activity was found in the soluble extract.

Cell cycle analysis showed TTL activity as two prominent peaks in V79 cells occurring at 4 and 7 h into the cycle. This corresponds to an early S and mid to late S peak. CHO cells displayed a major peak of activity at 9.5 h into the cycle just before mid S. In addition to the major peak of TTL activity at 9.5 h, there was a minor peak or plateau at 4–5 h into the cycle. The data indicate that TTL activity increases during G1 and reaches a maximum in S which is two–three times the G1 level and then decreases during late S and G2 to a minimum at mitosis. The oscillatory pattern of this enzyme was similar to other protein patterns that have been described in Chinese hamster cells (9, 13, 17, 20).

The implication that TTL enzyme may have some regulatory function on tubulin in the cell cycle prompted us to look at the relationship between TTL and tubulin as measured by colchicine binding. Our previous work showed that tubulin in Don and V79 cells was degraded during S phase and synthesized mostly in G2 just before mitosis (13). The data presented here indicate that tubulin in CHO cells is synthesized throughout the cell cycle with a major burst of synthesis in G2. This is in agreement with the data presented by Nolan et al. (27). The maximum in colchicine binding occurred when TTL activity was rapidly declining to its minimal level. This shows an out of phase relationship between tubulin synthesis and TTL activity.

The effect of tyrosylation on tubulin polymerization appears to be minimal in vitro. Raybin and Flavin could not demonstrate any effects in vitro on the tubulin polymerization reaction from calf brain (32). However, this does not rule out any effects in vivo where the milieu may be quite different or other effects in vitro since only polymerization was tested. In most cases, TTL activity is highest in neural tissues where tubulin is also found in high concentrations (4, 10, 32). However, during early development in chick muscle tissue, TTL activity is higher than in chick brain (10).

Raybin and Flavin have suggested that tyrosine may not be the only substrate which is utilized in vivo. They have demonstrated competitive inhibition of tyrosine by dipeptides containing tyrosine (33). The time of maximum TTL activity during the cell cycle corresponds closely to the time of maximum tubulin degradation during S phase (13). By attaching a peptide marker to tubulin at a specific time during the cell cycle, the cell might use this marker as a signal to either degrade the protein or to transport it to some other intracellular area. We might better be able to answer some of these questions by further analyzing the changes in tubulin through the cell cycle and determining its ability to be tyrosylated at different cell cycle times.

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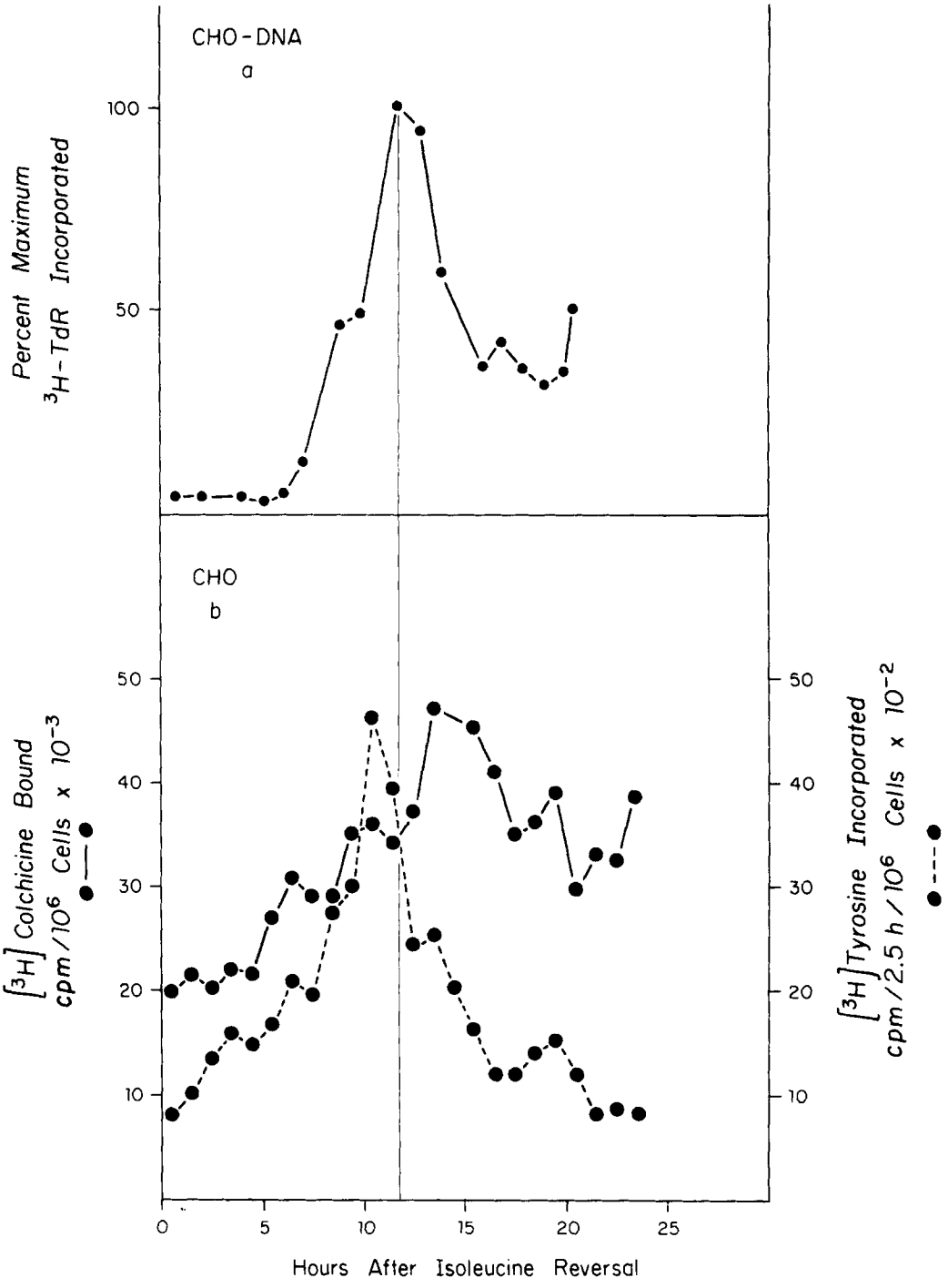

FIGURE 6 TTL and colchicine-binding activity through the CHO cell cycle. CHO cells were synchronized by isoleucine deprivation. (*a*) DNA in CHO cells was labeled with ^{3}H-TdR for 1 h at hourly intervals after isoleucine reversal. (*b*) After reversal, samples were taken every hour and analyzed for [^{3}H]colchicine binding (●——●) and for TTL activity (●- - -●).

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