Multiple Forms of Tubulin in *Polytomella* and *Chlamydomonas*: Evidence for a Precursor of Flagellar α-Tubulin

TIMOTHY W. McKEITHAN, PAUL A. LEFEBVRE, CAROLYN D. SILFLOW, and JOEL L. ROSENBAUM

Department of Biology, Yale University, New Haven, Connecticut 06511. Dr. McKeithan's present address is Department of Pathology, University of Chicago, Chicago, Illinois 60637. Dr. Lefebvre's and Dr. Silflow's present address is Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108.

ABSTRACT The quadriflagellate alga *Polytomella agilis* contains several α-tubulins with distinct isoelectric points (McKeithan, T. W., and J. L. Rosenbaum, 1981, *J. Cell Biol.*, 91:352–360). While α-3 is the major component in flagella, α-1 predominates in cytoskeletal microtubules. For determination of whether the differences in α-tubulins are due to distinct genes or to posttranslational modification of a common α-tubulin precursor, poly A+ RNA was isolated from deflagellated and control (nonregenerating) cells and translated in vitro. Approximately twice as much α-1 was synthesized using RNA from deflagellated as compared to control cells; however, there was no detectable synthesis in vitro of α-3 in either. These results suggest that α-3 tubulin is formed in vivo by posttranslational modification of a form co-migrating with, and possibly identical to, cytoskeletal α-tubulin. In the related alga *Chlamydomonas reinhardii* deflagellation greatly stimulates synthesis of tubulin and tubulin mRNA. As in *Polytomella*, the principal α-tubulin synthesized both in vivo and in vitro following deflagellation in *Chlamydomonas* is more basic than the major α-tubulin and appears to correspond to α-1 tubulin in *Polytomella*. The conversion of α-1 to α-3 receives additional support from in vivo labeling and pulse-chase experiments. In addition, in both *Polytomella* and *Chlamydomonas* some conversion of α-1 to α-3 appears to occur even when protein synthesis is inhibited.

Several functionally and structurally distinct microtubule organelles co-exist in many cell types. The mechanism by which specific protein components are assembled into appropriate microtubules and are excluded from other microtubules in the cell is not known. It is possible that in some cases the tubulin components of specific microtubule organelles may be distinct and may contribute to determining the other protein components of the microtubule.

We have shown (17) in the unicellular colorless alga *Polytomella agilis* that the major α-tubulins of cytoskeletal and flagellar microtubules are different. There is evidence in other systems that separate forms of tubulin are associated with specific microtubule organelles. Stephens (26–28) has evidence that different microtubule components of axonemes from sea urchin sperm or cilia are composed of tubulins with distinct amino acid compositions and peptide maps. An antibody against flagellar outer doublet tubulin in *Naegleria gruberi* appears not to cross-react with cytoplasmic tubulin of the same cell, and pulse-chase experiments also suggest that newly synthesized outer doublet tubulin does not exchange with the preexisting cyotplasmic tubulin pool (11, 12).

In several other systems, α- or β-tubulin has been shown to be modified after translation. Brain β-tubulin is specifically phosphorylated (7) while tyrosine is removed from a portion of brain α-subunits by a tubulin-specific carboxypeptidase (13). A separate ATP-requiring tubulin tyrosyl-ligase has been characterized which adds tyrosine to the C-terminus (1–3, 8, 20). A single gene appears to be responsible for two distinct β-subunits

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 APRIL 1983 1056–1063
 · 0021-9525/83/04/1056/08 $1.00

in *Aspergillus* (23). On the basis of these results in other systems, we attempted to determine whether tubulin heterogeneity in *Polytomella* is due to posttranslational modification of a single precursor.

The present paper presents evidence from both in vivo labeling and in vitro translation studies showing that flagellar α-3 tubulin in both Chlamydomonas and *Polytomella* is synthesized as a more basic precursor which in *Polytomella* comigrates with cytoskeletal α-1 tubulin. Pulse-chase experiments in *Chlamydomonas* also support the model of conversion of α-1 to α-3, as do preliminary experiments which appear to show conversion of α-1 to α-3 in the absence of protein synthesis.

MATERIALS AND METHODS

Cell Cultures: Axenic cultures of *P. agilis* were grown as previously described (17). Large volumes were grown with gentle aeration. Most experiments on *Chlamydomonas reinhardi* used the cell wall-less mutant CW15 (mating type −), which was grown as previously described (16).

In Vivo Labeling: One-dimensional Gels: 750 ml of a culture of *Polytomella* grown to ~1 × 10^6 cell/ml were pelleted at low speed and transferred sterilely to an equal volume of medium lacking sodium acetate, the principal energy source, and having all sulfate replaced by chloride, after being washed once in the sulfur-free medium. After starvation for 17 h, cells were deflagellated by vortexing in a fluted tube for 1 min (5, 22), and 5-ml aliquots were labeled at 30-min intervals with 0.5 mCi $^{35}SO_4$ (43 Ci/mg, carrier free) (New England Nuclear, Boston, MA). Samples were pelleted at low speed, resuspended in water, and boiled for 2 min with an equal volume of SDS sample buffer (14).

In Vivo Labeling: Two-dimensional Gels: Samples for two-dimensional (2-D) electrophoresis were prepared as previously described for *Chlamydomonas* (16) and *Polytomella* (17), except that in most cases after suspension in 2-D sample buffer the samples were centrifuged at 106,000 *g* for 60 min at 25°C (Beckman L2-65B centrifuge, Ti 50 rotor, 40,000 rpm) and the supernates stored at −70°C until use. Acetone precipitation of samples of *Chlamydomonas* before preparation for 2-D electrophoresis was necessary to prevent severe streaking and artefactual spots.

One- and Two-dimensional Gel Analysis: Samples for electrophoresis on one-dimensional (1-D) slab gels were run on 6–16% acrylamide gradient SDS gels made with a 3–8 M gradient of urea, as described (15). 2-D gel electrophoresis was performed as described by O'Farrell (19), with minor modifications (16, 17). The methods for gel staining, autoradiography and fluorography have been described (15, 16).

RNA Isolation and In Vitro Translation: Total RNA and poly(A) RNA were prepared from *Polytomella* which had been starved for acetate overnight as described above. RNA was isolated from nondeflagellated cells and from cells 45 min after deflagellation. RNA isolation, in vitro translation, and immunoprecipitation were done as previously described (16, 25).

Pulse-Chase Experiments: A culture of the CW15 strain of *Chlamydomonas,* starved overnight for sulfur, was pelleted and resuspended in about one-fourth volume of the initial growth medium. The cells were deflagellated by pH stock (22), pelleted, and resuspended in a portion of the initial growth medium. At 6.5 min after deflagellation, 0.4 mCi/ml $^{35}SO_4$ was added to a portion of the deflagellated cells; the cells were pelleted and resuspended in "chase" medium with cold sulfate (6 mM) at 24 min after deflagellation. Samples were prepared at 50, 75, and 100 min following deflagellation. Three samples of cells, from the same deflagellated sample, but not initially labeled, were labeled from 25 to 50 min, 50 to 75 min, and 75 to 100 min following deflagellation (corresponding to 0–25 min, 25–50 min, and 50–75 min of the chase period of the pulse-chase sample).

Cycloheximide Experiments: A culture of *Chlamydomonas* CW15 was grown 4 d in $^{35}SO_4$ in low sulfur medium, pelleted, and resuspended in medium containing 10 μg/ml cycloheximide. A portion was immediately prepared for 2-D electrophoresis. Another portion was deflagellated by pH stock, and an aliquot of deflagellated cells prepared for 2-D electrophoresis immediately after deflagellation. The remainder was allowed to regenerate flagella for 45 min. A portion of this sample was deflagellated again by pH shock. Samples were prepared for 2-D electrophoresis as described above.

A culture of *Polytomella* was washed in sulfur-free medium; after 2 h in sulfur-free medium, 0.2 mCi/ml $^{35}SO_4$ was added. After 45 min, cycloheximide was added to 20 μg/ml. After 25 min more the sample was washed twice with sulfur-free medium containing cycloheximide and one-third of the sample was prepared for 2-D electrophoresis. After 10 min more the remaining sample was deflagellated by vortex agitation, and one-half of the cell bodies pelleted and prepared for 2-D electrophoresis. The remaining sample was allowed partially to regenerate flagella and after one hour was pelleted and prepared for 2-D electrophoresis.

RESULTS

In *P. agilis* the principal flagellar alpha tubulin (α-3) is clearly distinct from the principal α-tubulin of cytoskeletal microtubules, α-1, which has a more basic pI (17). In the related organism *Chlamydomonas* a similar set of α-tubulins is found, and evidence has been presented suggesting that α-1 may be a precursor of α-3 (16). Experiments were designed to bring together these two separate lines of research to develop a coherent model encompassing both organisms.

Stimulation of Tubulin Synthesis following Deflagellation

The presence of multiple tubulins in *Polytomella* raises the question of whether these different tubulins are produced from distinct genes or instead are the products of posttranslational modification of a common precursor. Detecting a precursor to a form of tubulin would be facilitated by finding conditions in which synthesis of that particular tubulin is specifically increased. Since in other systems flagellar protein synthesis and, in particular, flagellar tubulin synthesis increases following deflagellation (15, 29), the response of flagellar tubulin synthesis to deflagellation was studied in *Polytomella.*

A culture of *Polytomella* was transferred to medium lacking sulfate and acetate for 17 h in order to starve the cells and reduce background tubulin synthesis. The cells were subsequently deflagellated and labeled with $^{35}SO_4$ at 30-min intervals. Fig. 1 is an SDS gel illustrating the experiment. The leftmost lane is a flagellar standard; the second lane is a nondeflagellated control sample; and subsequent lanes show successive 30-min labeling intervals following deflagellation. The synthesis of several flagellar proteins increases following deflagellation (Fig. 1, arrows), including that of α- and β-tubulin and the high molecular weight dyneins.

The additional tubulin synthesized following deflagellation was presumed to represent flagellar tubulin, and the bulk of the tubulin synthesized in nondeflagellated control cells was presumed to be cytoskeletal, since the latter is the principal tubulin in the cell. To determine whether in either case the tubulin is synthesized as a precursor, we isolated mRNA for in vitro translation, since posttranslational modifications would be less likely to occur in vitro. Total RNA was extracted from nondeflagellated cells and from cells 45 min after deflagellation, and poly(A+)RNA was isolated by chromatography on oligo(dT) cellulose. Translation of the RNA in the reticulocyte lysate system was followed by immunoprecipitation of the tubulins using antibody prepared against α- and β-tubulin isolated from *Chlamydomonas* flagellar axonemes by preparative 2-D electrophoresis (25).

The translation products of poly(A+)RNA of nondeflagellated cells (*N*) and of deflagellated cells (*D*) are displayed in Fig. 2*a,* lanes *2* and *3.* Proteins as large as the dyneins (~350,000 mol wt) appear to be synthesized in vitro. Both tubulin subunits are undertranslated in vitro. A few flagellar proteins can be seen to increase in the deflagellated sample, including α- and β-tubulin and dynein (Fig. 2*a,* arrows). Lanes *6–9* (Fig. 2*a*) represent immunoprecipitates prepared from translation samples with equal numbers of total incorporated radioactive counts. The quantity of translatable mRNA for both α- and β-tubulin is greater in the samples from deflagel-

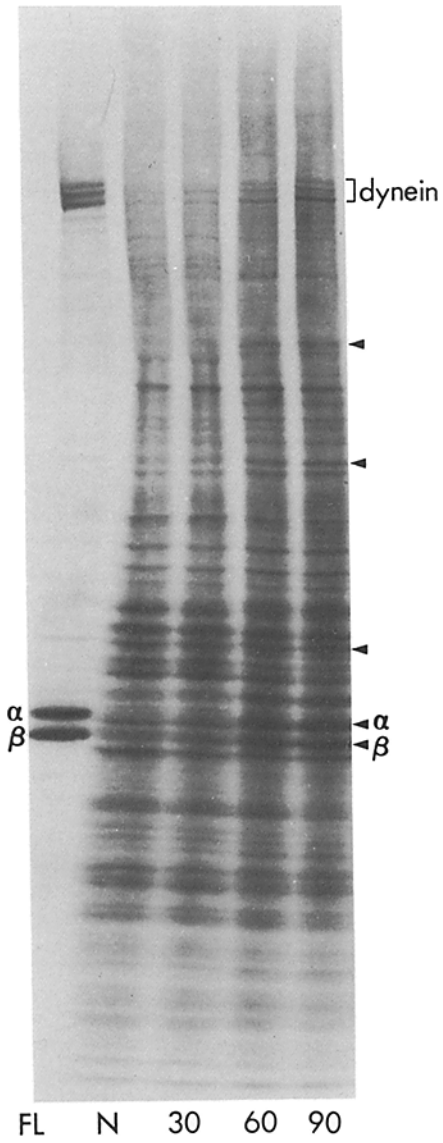

FIGURE 1 Autoradiograph of SDS gel of whole *Polytomella* labeled with 30-min pulses of $^{35}SO_4$. Lane *FL* represents a flagellar standard; lane *N*, a nondeflagellated sample; and the following three lanes, samples labeled in pulses ending at 30, 60, and 90 min following deflagellation. α- and β-tubulins are marked by arrowheads.

lated cells (Fig. 2*a*, lanes *7* and *9*) than in samples from nondeflagellated controls (Fig. 2*a*, lanes *6* and *8*). As judged by total counts immunoprecipitated, α-tubulin mRNA increases 95%, and β 64%, following deflagellation.

The radioactively labeled translation products of these two RNA samples were mixed with unlabeled tubulin standards and subjected to 2-D electrophoresis. Autoradiographs of the 2-D gels of the translation products (Fig. 2*d* and *e*) show, by comparison with the stained gels showing the tubulin standards (*insets* to Fig. 2*d* and *e*), that the α-tubulin made in vitro is α-1, the predominant form of tubulin in whole cell homogenates (Fig. 2*b*); little or no α-3, the major flagellar tubulin (Fig. 2*c*), is synthesized, even in the deflagellated sample. Since the increased tubulin synthesis is the result of deflagellation, this additional tubulin synthesis is likely to represent flagellar tubulin. These results suggested the possibility that α-3, the principal flagellar α-tubulin, is produced by posttranslational modification of α-1, the tubulin spot characteristic of cytoskeletal tubulin, since α-1 increases with deflagellation but α-3 is not synthesized in detectable amounts.

Consistent with these in vitro labeling results, in experiments similar to that shown in Fig. 1, 2-D electrophoresis of pulse-labeled cells following deflagellation failed to reveal any significant increased synthesis of α-3 tubulin, the predominant α-tubulin present in flagellar axonemes (results not shown).

Since α-1 tubulin migrates slightly faster than α-3 in standard SDS gels, even 1-D gels can in some cases provide information concerning the form of α-tubulin present. Careful examination of 1-D SDS gels such as in Fig. 1 shows the α-tubulin whose synthesis increases migrates more rapidly than the α-tubulin of adjacent flagellar standards, suggesting that synthesis of α-1, not of α-3, is stimulated by deflagellation.

Since in *Polytomella* the large quantity of cytoskeletal tubulin may tend to obscure processing of flagellar tubulin, further experiments were performed in the related organism *Chlamydomonas* which has comparatively very few cytoplasmic microtubules in interphase. As previously reported, *Chlamydomonas* contains two major α-tubulins which appear to correspond to α-1 and α-3 tubulin in *Polytomella*. Deflagellation results in greatly increased synthesis of tubulins, and the principal alpha tubulin synthesized in vivo is α-1, not α-3, the major flagellar α-tubulin. In vitro translation of isolated RNA yields the same result (16). This corresponds to the results reported here in *Polytomella* and suggests that α-1 may be a precursor to α-3.

Pulse-Chase Experiments

Pulse-chase experiments were performed to determine whether α-1 is the precursor to α-3 in vivo in *Chlamydomonas*. Deflagellated cells were labeled with an 18-min pulse of $^{35}SO_4$ and a sample was processed for gel electrophoresis; the remaining cells were subjected to a chase with cold sulfate. Samples were taken after ~25, 50, and 75 min of chase. Radioactive counts at the relevant time points showed that the chase was effective, and, although there was some continued net radioactive incorporation during the first chase time period, there was no net incorporation at later intervals. As a control, a parallel sample of deflagellated cells was labeled during each of the chase time periods. Each sample was subjected to 2-D electrophoresis together with unstained tubulin standards; tubulin spots were identified in the autoradiographs by comparison with the unlabeled standards in the stained gel (illustrated in the *inset* to Fig. 3*b*).

Following deflagellation (Fig. 3*b*), there is a dramatic increase in the quantity of α-1 and β-tubulin synthesis compared to that of nondeflagellated cells (Fig. 3*a*); in order for the tubulin spots not to be overexposed in Fig. 3*b–h* a relatively much shorter exposure is required than in Fig. 3*a*, so that background spots are not seen in the later panels. Little α-3, the major flagellar α-tubulin, is initially synthesized following deflagellation. In the chase samples (Fig. 3*c, d,* and *e*), the ratio of α-3 to α-1 is seen to increase steadily during the chase, consistent with a conversion of α-1 to α-3. Since the chase was not totally effective, radioactive incorporation into tubulin likely occurred during at least the first chase period. To rule out the possibility that α-3 *de novo* synthesis is occurring during the chase period, we examined the control samples pulse-labeled during the chase periods (Fig. 3*f, g,* and *h*). In each of the periods the synthesis of α-1 in the control samples was greater than synthesis of α-3, showing that a late turn-on of

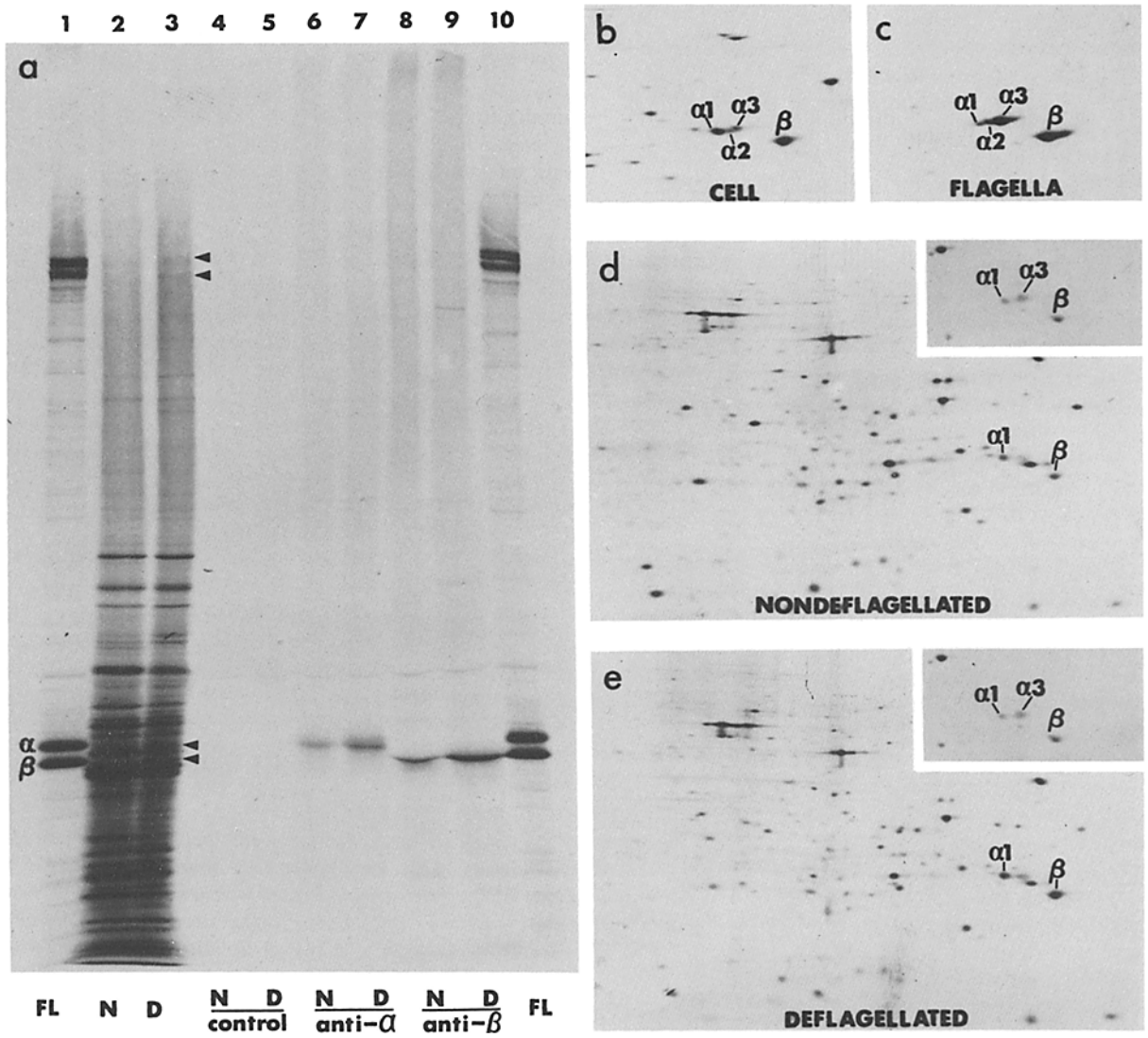

FIGURE 2 (*a*) An autoradiograph of an SDS gel illustrating immunoprecipitation of in vitro translation products from RNA isolated from *Polytomella*. Lane *FL* represents flagellar standard; lanes *N* and *D*, that of translation products from RNA isolated from nondeflagellated and deflagellated cells. Lanes *4* and *5*, *6* and *7*, and *8* and *9* are of immunoprecipitates made using preimmune serum, anti-α-tubulin serum, and anti-β-tubulin serum, respectively. (*b* and *c*) The tubulin regions of 2-D gels of whole *Polytomella* cells and flagella, respectively, demonstrating that α-3 is the principal alpha tubulin in flagella, and α-1, the principal α-tubulin in whole cells. (*d* and *e*) Autoradiographs of 2-D gels of in vitro translation products using RNA isolated from nondeflagellated and deflagellated cells, respectively. *Insets* show the Coomassie Blue staining pattern of the tubulin regions of the same 2-D gels, which were run with cold standard consisting of a mixture of cytoskeletal tubulin and of flagella to show the positions of α-1 and α-3 tubulin.

α-3 synthesis was not responsible for the increase in α-3 in comparison to α-1 seen in the experimental cell sample during the chase. The small amounts of α-3 present in the control samples may be explained by the long pulse periods, which may have permitted some conversion to occur.

Conversion of α-1 to α-3 in the Absence of Protein Synthesis

Experiments were performed to determine whether conversion of α-1 to α-3 can occur in the absence of protein synthesis (Fig. 4). Both *Chlamydomonas* and *Polytomella* can partially regenerate their flagella in the absence of protein synthesis (5, 21). For experiments with *Polytomella*, cells were transferred to medium without acetate or sulfate for 2 h and labeled for 45 min with $^{35}SO_4$. The cells were then placed in 20 μg/ml cycloheximide to inhibit protein synthesis, and after 20 min a portion of the cells was prepared for 2-D electrophoresis (Fig. 4*a*) while the remaining cells were deflagellated. (In control experiments, this concentration of cycloheximide inhibited protein synthesis by at least 96%.) A portion of the cells was immediately prepared for 2-D electrophoresis (Fig. 4*b*) while another portion was first allowed to regenerate flagella for 45 min (Fig. 4*c*). 2-D electrophoresis of the samples shows that, while essentially no α-3 tubulin was present in cells deflagellated and immediately prepared for electrophoresis (Fig. 4*b*), regenerated cells showed an α-3 spot (Fig. 4*c*). Since α-3 was formed in the absence of significant protein synthesis, it presumably is derived from a precursor. Since the quantity of cytoskeletal microtubules in *Polytomella* is much greater than the quantity of flagellar microtubules, even after regeneration, the α-3 tubulin spot is much less prominent than the α-1 spot.

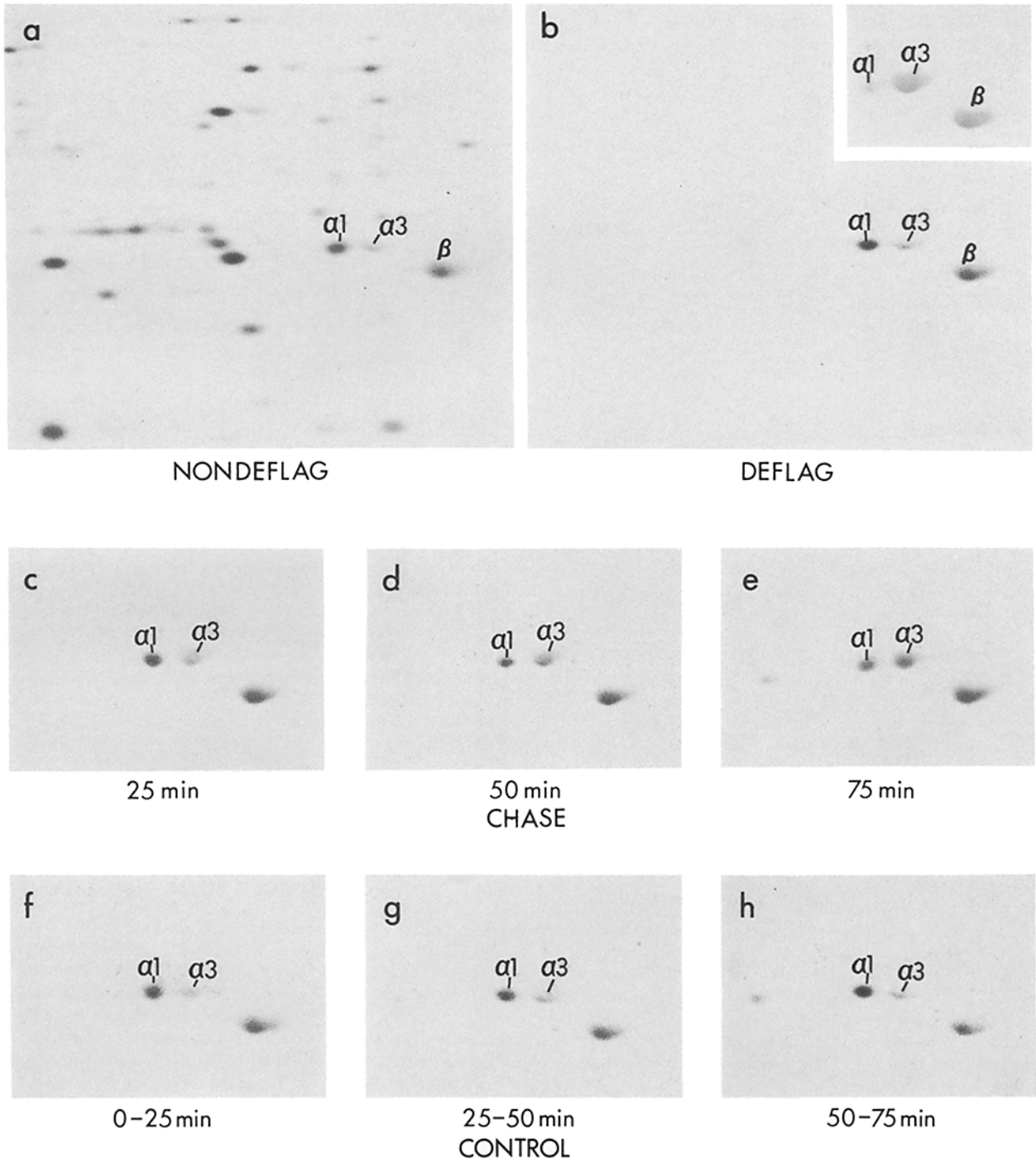

FIGURE 3 Pulse-chase experiment in *Chlamydomonas*. (*a*) An autoradiogram of the tubulin region of pulse-labeled nondeflagellated cells (a separate experiment from the following samples). (*b*) An autoradiograph of cells pulse-labeled following deflagellation. (*c*, *d*, and *e*) Autoradiographs of samples chased with cold sulfate for an additional 25, 50, and 75 min, respectively. (*f*, *g*, and *h*) Control samples deflagellated at the same time as the experimental group but labeled for 25-min intervals corresponding to the chase periods of the pulse-chase samples.

In *Chlamydomonas* a similar experiment was performed using uniformly ^{35}S-labeled cells. Cycloheximide was used at 10 μg/ml, which has been shown to inhibit protein synthesis in *Chlamydomonas* by at least 98% (21). Samples were prepared before deflagellation (Fig. 4*d*), immediately following deflagellation (Fig. 4*e*), and after partial flagellar regeneration in the presence of cycloheximide (Fig. 4*f*). In addition, a portion of the cells with partially regenerated flagella was deflagellated again and the cell bodies prepared for electrophoresis (Fig. 4*g*). Following deflagellation, the intensity of the α-3 spot diminishes (Fig. 4*e*). The position of the remaining material at the isoelectric point of α-3 does not superimpose on the stained spot of the cold flagellar sample which was included in the electrophoresis but instead forms a thin crescent at the front of the stained spot. Following partial flagellar regeneration in cycloheximide (Fig. 4*f*), there appears to be a small decrease

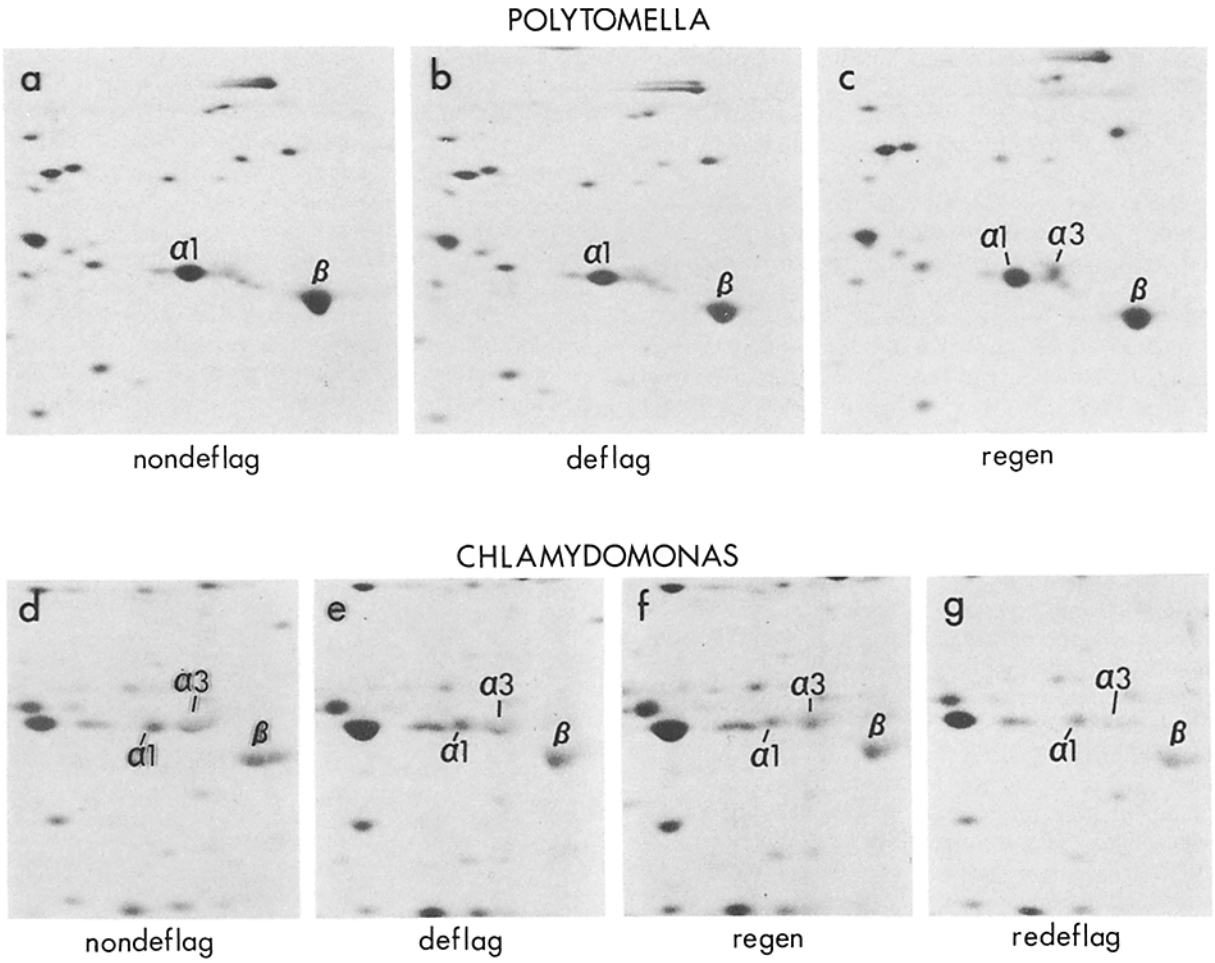

FIGURE 4 Experiments with cycloheximide. The upper three panels show samples of *Polytomella* labeled for 45 min with $^{35}SO_4$ followed by inhibition of protein synthesis with cycloheximide: (*a*) nondeflagellated sample; (*b*) sample prepared immediately after deflagellation; and (c) sample allowed partially to regenerate flagella in the absence of protein synthesis. The four lower panels show uniformly labeled samples of *Chlamydomonas; (d)* nondeflagellated sample; (e) sample prepared immediately after deflagellation; (*f*) sample allowed partially to regenerate flagella under cycloheximide inhibition; (*g*) sample prepared immediately after a second deflagellation following partial flagellar regeneration.

in the quantity of the α-1 spot and an increase in α-3 labeling. This suggests that there is formation of α-3 without protein synthesis. The change is relatively small, suggesting that part of the α-1 may be modified before the cells are deflagellated. The material in the deflagellated but unregenerated sample at the pI of α-3 (Fig. 4*e*) appears to migrate slightly faster in the SDS dimension than α-3, suggesting that little bona fide α-3 is present before regeneration. Most of the α-3 spot is lost after the cells are again deflagellated (Fig. 4*h*); this result is expected since the cells are incapable of further flagellar regeneration.

DISCUSSION

Several types of experiments provide evidence that in *Polytomella* and in *Chlamydomonas* α-3 tubulin is formed from a precursor which migrates at the position of α-1, which in *Polytomella* is the principal α-tubulin of cytoskeletal microtubules. In both species, following deflagellation the principal α-tubulin synthesized is α-1 tubulin, both in vivo and in translation of isolated RNA in vitro. Pulse-chase experiments in *Chlamydomonas* show apparent conversion of α-1 to α-3, and preliminary experiments using cycloheximide appear to show conversion of α-1 to α-3 in the absence of protein synthesis.

If α-1 is the precursor to α-3, one would expect no α-3 to be present in the products of in vitro translation of cell RNA. However, in translation of *Chlamydomonas* RNA isolated from deflagellated cells, a small amount of label is found in the area of α-3 (16), and this spot, as well as the α-1 spot, is precipitated by the α-tubulin antibody (24). In gels in which unlabeled flagellar standard has been included in the electrophoresis, the label forms a crescent at the bottom of the α-3 spot but does not completely overlap with the stained spot showing the cold flagellar standard. α-1 migrates very slightly faster than α-3 on SDS gels, and in 2-D gels which show streaking the streak from alpha-1 extends below the α-3 spot. This fact suggests that the label found near the α-3 spot in gels of in vitro translation products is not bona fide α-3 but instead is derived from α-1 either by artefactual modification or by modification in the in vitro translation system.

In one case a spot which approximately co-migrates with the α-3 spot is found in 2-D gels of in vitro translation products; this has so far only been found in *Chlamydomonas* in cell samples during or after induction of flagellar resorption. Since flagellar resorption causes a reduction in synthesis of many flagellar proteins and an overall decrease in tubulin synthesis, it is unlikely that the protein spot synthesized under these

conditions is a flagellar protein. It is possible that conditions which result in flagellar resorption also cause the synthesis of tubulins which are specific for other microtubular organelles, such as the mitotic apparatus, the basal bodies, or the cytoplasmic rootlets and that this tubulin fortuitously co-migrates with flagellar α-3 tubulin.

Using cloned probes containing α-tubulin DNA sequences, we and others have previously shown that there are at least two α-tubulin genes in *Chlamydomonas* (6, 25). In addition, two mRNA species were detected in approximately equal quantities. The quantity of both forms of mRNA increased coordinately following deflagellation and decreased under flagellar resorption conditions (24). The two mRNA species appear to correspond to the two α-tubulin genes which have been detected (6; and our unpublished observations). The fact that α-1 constitutes all or almost all of the α-tubulin synthesized in vitro using deflagellated cell RNA suggests that both mRNA species code for α-1. To test this hypothesis, experiments are in progress to isolate and translate in vitro each of the two α-tubulin mRNA's.

In *Polytomella* the apparent precursor to α-3 tubulin co-migrates with α-1, the principal α-tubulin of the cytoskeletal microtubules (17). It has not been determined whether, in fact, the precursor is identical to cytoskeletal α-1. If it is identical, then a single tubulin species can assemble into two structurally quite different forms of microtubule.

In some cases there has been circumstantial evidence for a common tubulin pool; for example, in many cells cytoplasmic or flagellar microtubules disassemble before formation of the mitotic spindle (4). Nevertheless, there is little *direct* evidence in vivo that tubulin from one microtubule-containing organelle can be used to build another type of organelle. A tubulin mutant has been isolated in *Drosophila* which affects only the testis-specific beta tubulin subunit (9, 10). The testis-specific subunit has been shown to be a component of sperm flagella. However, in the mutant the meiotic spindle is disrupted, presumably as a result of incorporation of the mutant tubulin into the spindle. Thus, at least in mutant insects a flagellar β-tubulin may also participate in formation of cytoplasmic singlet microtubules. In another case, mutants in the Ben A locus coding for β-tubulin in *Aspergillus* appear to be altered in both nuclear division, involving mitotic microtubules, and in nuclear migration, which depends on cytoplasmic microtubules. Both processes are also affected by a different mutation in a structural gene for α-tubulin (18).

The mechanism is not known by which, in cells which have several microtubule organelles composed of distinct tubulins, only the appropriate form of tubulin is incorporated into each microtubule. In *Polytomella* and *Chlamydomonas* the possibility exists that the modification of α-1 to α-3 occurs during or after addition of the tubulin subunit onto the flagellar microtubule. If this possibility is correct, then a growing microtubule is not required to distinguish among tubulin dimers. If this modification occurs in conjunction with flagellar assumbly, it may be an important site for control of flagellar assembly.

If the cell maintains little or no monomeric pool of α-3 tubulin, the other microtubule organelles will not be "contaminated" with flagellar α-tubulin. If this is an important function of restricting α-3 tubulin to the flagella, one would expect in *Chlamydomonas,* which can be induced to resorb its flagella, that the modification might be reversed during flagellar resorption. Recent experiments have shown that in conjunction with flagellar resorption there is a decrease in the label in α-3 tubulin and an increase in the label in α-1 tubulin (S. L'Hernault, manuscript submitted); these results suggest that the modification of α-1 to α-3 which occurs during regeneration can be reversed by resorption.

The nature of the modification of α-1 to α-3 has not been determined. Experiments using labeling with $^{32}PO_4$ have failed to show labeling of α-3, although several other flagellar proteins are labeled; thus, phosphorylation is apparently not the modification involved. Possibilities include sulfation, deamidation, and acylation of the N-terminus or of a lysine residue. The removal of carboxy-terminal tyrosine which occurs in brain tubulin should not result in change in protein charge, which is required for modification of α-1 to α-3. Experiments involving peptide mapping are in progress to determine the nature of the protein modification.

We would like to thank Steven L'Hernault for stimulating discussions, and Ditti Holub for excellent technical assistance.

This work was suported by U. S. Public Health Service (USPHS) grant GM 14,642 to J. L. Rosenbaum and by the Insurance Medical Scientist Scholarship Fund (Hartford Insurance Group, sponsor) and USPHS predoctoral fellowship GM02044 to T. W. McKeithan.

Received for publication 30 August 1982.

REFERENCES

1. Arce, C. A., J. A. Rodriguez, H. S. Barra, and R. Caputto. 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.
2. Argarana, C. E., C. A. Arce, H. S. Barra, and R. Caputto. 1977. *In vivo* incorporation of ^{14}C-tyrosine into the C-terminal position of the alpha subunit of tubulin. *Arch. Biochem. Biophys.* 180:264:268.
3. Barra, H. S., C. A. Arce, J. A. Rodriguez, and R. Caputto. 1974. Some common properties of the protein that incorporates tyrosine as a single unit and the microtubule proteins. *Biochem. Biophys. Res. Commun.* 60:1384–1390.
4. Bloodgood, R. A. 1974. Resorption of organelles containing microtubules. *Cytobios.* 9:143–161.
5. Brown, D. L., and K. A. Rogers. 1978. Hydrostatic pressure-induced internalization of flagellar axonemes, disassembly, and reutilization during flagellar regeneration in *Polytomella. Exp. Cell Res.* 117:313–324.
6. Brunke, K. J., E. E. Young, B. U. Buchbinder, and D. P. Weeks. 1982. Coordinate regulation of the four tubulin genes of *Chlamydomonas reinhardi. Nucleic Acids Res.* 10:1295–1310.
7. Eipper, B. A. 1974. Rat brain tubulin and protein kinase activity. *J. Biol. Chem.* 249:1398–1406.
8. Hallack, M. E., J. A. Rodriguez, H. S. Barra, and R. Caputto. 1977. Release of tyrosine from tyrosinated tubulin. Some common factors that affect this process and the assembly of tubulin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 73:147–150.
9. Kemphues, K. J., E. C. Raff, R. A. Raff, and T. C. Kaufman. 1980. Mutation in a testis-specific beta-tubulin in *Drosophila*: analysis of its effects on meiosis and map location. *Cell.* 21:445–451.
10. Kemphues, K. J., R. A. Raff, T. C. Kaufman, and E. C. Raff. 1979. Mutation in a structural gene for a beta-tubulin specific to testis in *Drosphila melanogaster. Proc. Natl. Acad. Sci. USA.* 76:3991–3995.
11. Kowit, J. D., and C. Fulton. 1974. Programmed synthesis of tubulin for the flagella that develop during cell differentiation in *Naegleria gruberi. Proc. Natl. Acad. Sci. USA.* 71:2877–2881.
12. Kowit, J. D., and C. Fulton. 1974. Purification and properties of flagellar outer doublet tubulin from *Naegleria gruberi* and a radioimmune assay for tubulin. *J. Biol. Chem.* 249:3638–3646.
13. Kumar, N., and M. Flavin. 1981. Preferential action of a brain detyrosinolating carboxypeptidase on polymerized tubulin. *J. Biol. Chem.* 256:7678–7686.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680–685.
15. Lefebvre, P. A., S. A. Nordstrom, J. E. Moulder, and J. L. Rosenbaum. 1978. Flagellar elongation and shortening in *Chlamydomonas.* IV. Effects of flagellar detachment, regeneration, and resorption on the induction of flagellar protein synthesis. *J. Cell Biol.* 78:8–27.
16. Lefebvre, P. A., C. D. Silflow, and J. L. Rosenbaum. 1980. Increased levels of mRNAs for tubulin and other flagellar proteins after amputation or shortening of *Chlamydomonas* flagella. *Cell.* 20:469–477.
17. McKeithan, T. W., and J. L. Rosenbaum. 1981. Multiple forms of tubulin in the cytoskeletal and flagellar microtubules of *Polytomella. J. Cell Biol.* 91:352–360.
18. Oakley, B. R., and N. R. Morris. 1981. A beta-tubulin mutation in *Aspergillus nidulans* that blocks microtubule function without blocking assembly. *Cell.* 24:837–845.
19. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
20. Raybin, D., and M. Flavin. 1975. An enzyme tyrosylating alpha-tubulin and its role in microtubule assembly. *Biochem. Biophys. Res. Commun.* 65:1088–1095.
21. Rosenbaum, J. L., J. E. Moulder, and D. Ringo. 1969. Flagellar elongation and shortening in *Chlamydomonas.* The use of cycloheximide and colchicine to study the synthesis and

assembly of flagellar proteins. *J. Cell Biol.* 41:600–619.
22. Rosenbaum, J. L., and F. M. Child. 1967. Flagellar regeneration in protozoan flagellates. *J. Cell Biol.* 34:345–364.
23. Sheir-Neiss, G., M. H. Lai, and N. R. Morris. 1978. Identification of a gene for beta-tubulin in *Aspergillus nidulans*. *Cell.* 15:638–647.
24. Silflow, C. D., P. A. Lefebvre, T. W. McKeithan, J. A. Schloss, L. R. Keller, and J. L. Rosenbaum. 1981. Expression of flagellar protein genes during flagellar regeneration in *Chlamydomonas*. *Cold Spring Harbor Symp. Quant. Biol.* 45:1912.
25. Silflow, C. D., and J. L. Rosenbaum. 1981. Multiple alpha- and beta-tubulin genes in *Chlamydomonas* and regulation of tubulin mRNA levels after deflagellation. *Cell.* 24:81–88.
26. Stephens, R. E. 1970. Thermal fractionation of outer fiber doublet microtubules into A- and B-tubulin. *J. Mol. Biol.* 47:353–363.
27. Stephens, R. E. 1975. Structural chemistry of the axoneme: evidence for chemically and functionally unique tubulin dimers in outer fibers. *In* Molecules and Cell Movement. S. Inoue and R. E. Stephens, editors. Raven Press, New York. 181–204.
28. Stephens, R. E. 1978. Primary structural differences among tubulin subunits from flagella, cilia, and the cytoplasm. *Biochemistry.* 14:2882–2891.
29. Weeks, D. P., and P. S. Collis. 1976. Induction of microtubule protein synthesis in *Chlamydomonas reinhardi* during flagellar regeneration. *Cell.* 9:15–27.