Podophyllotoxin-resistant Mutants of Chinese Hamster Ovary Cells

ALTERATION IN A MICROTUBULE-ASSOCIATED PROTEIN*

(Received for publication, September 8, 1981)

Radhey S. Gupta†, Tommy K. W. Ho, Malcolm R. K. Moffat, and Rajni Gupta

From the Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Mutants of Chinese hamster ovary cells which have been obtained after one or two selection steps in the presence of the microtubule inhibitor podophyllotoxin (PodR and PodR* mutants, respectively) and which exhibit very specific types of cross resistance (and/or collateral sensitivity) towards various other inhibitors of microtubule assembly (e.g. colchicine, colcemid, stegnacin, vinblastine, nocodazole, griseofulvin, maytansine, taxol, and no cross-resistance to VM-26 and puroromycin) have been analyzed by two-dimensional gel electrophoresis. In two of the four PodR* mutants, a new protein spot, designated M (Mr = 66,000), is observed. Analyses of peptides obtained after partial protease digestion suggest that protein M has arisen by a charge alteration in a neighboring, more basic protein (P) found in the wild type cells. In the two PodR* mutant cells, the P and M proteins were present in a ratio of 1:0.8, whereas in cell hybrids formed between PodR* x PodR cells, their relative amounts were only about 1:0.25. This effect of gene dosage on the relative amounts of P and M proteins is consistent with the co-dominant nature of the PodR* mutation and indicates that in the mutant cells, only one of the two copies of the P gene is altered. The protein affected in these mutants appears to represent one of the microtubule-associated proteins by two criteria: (i) this protein (P in the case of sensitive cells or both P and M in the mutant), along with tubulin, is released from microtubules upon cold treatment; and (ii) this protein is released by Ca2+ treatment of the detergent-extracted cytoskeletons prepared from normal cells but not from colchicine-pretreated cells (Duerr, A., Pallas, D., and Solomon, F. (1981) Cell 24, 203–211). The relative intensities of different spots in gel patterns further suggest that protein P is a major cellular constituent present in cell extracts in amounts comparable to tubulin.

Microtubules are hollow filamentous structures, ubiquitous in eukaryotic cells, which form a cytoplasmic network, and are primarily responsible for determining cellular shape and for spindle formation required for cell division (see Refs. 1–3). In addition, microtubules play an important role in several other cellular processes, including motility, axonal growth, secretion (and transport), and cellular anchorage. Two closely related proteins, α- and β-tubulins, which have been highly conserved during evolution, are the major constituents of all microtubule structures. Recent work on the formation of microtubules in vitro indicates that several other proteins are also required in the assembly process (4–7). However, the involvement and precise role of these other proteins in microtubule structure in vivo is uncertain (8).

The assembly of microtubules both in vivo and in vitro is blocked by a number of inhibitors, of which colchicine, which binds to the tubulin dimer, is the best characterized (2, 9). The mechanisms of action of other inhibitors of microtubule assembly, such as vinblastine, podophyllotoxin, griseofulvin, nocodazole, maytansine, taxol, etc., are not so well understood (2, 9, 10, 11). Therefore, the sites of action of these drugs could possibly identify additional structural components involved in microtubule assembly or perturbation. The genetic approach, in which resistant mutants are isolated and the affected components are identified (12–15), has proved particularly useful in identifying the sites of action of various inhibitors. It has recently been shown that many of the mutants of CHO cells which have simultaneously become resistant to colchicine, colcemid, and griseofulvin (16), and mutants of Aspergillus nidulans resistant to benomyl (benzimidazole class of antimitotic drugs (17)) show alteration in the β-tubulin structure.

We have recently reported that mutants of CHO cells and several other mammalian cell lines (18, 19) showing 2-4-fold resistance to podophyllotoxin (PodR class), another antimitotic drug, can be readily selected in a single step. Mutants showing a higher level of resistance to the drug were also obtained in a second step selection using the PodR* mutants (PodR* class). Preliminary cross-resistance studies (18) indicated that these mutants were distinct from the colchicine and colcemid resistant mutants that had been obtained by other investigators, and that the lesions did not involve permeability alterations (16, 18, 21, 22). Cross-resistance studies reported here reveal that the PodR* and PodR* classes of mutants exhibit very specific types of cross-resistance (and/or collateral sensitivity) to various other inhibitors of microtubule assembly, e.g. colchicine, colcemid, vinblastine, griseofulvin, stegnacin, nocodazole, maytansine, and taxol. Based on their cross-resistance patterns, the mutants appear to be of more than one kind. The present paper also examines the nature of biochemical alteration in several PodR* and PodR* mutants by two-dimensional gel electrophoresis. The lesions in two of the PodR* mutants affect a protein with an approximate molecular weight of 66,000, which very likely represents one of the microtubule-associated proteins.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The Chinese hamster ovary cell lines shown in Table I were routinely grown in monolayer cultures

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by grants from the Medical Research Council of Canada during the tenure of a Research Career scholarship award. To whom correspondence should be addressed.

1 The abbreviations used are: CHO, Chinese hamster ovary; PodR*, mutants of CHO cells obtained after n selection steps in the presence of podophyllotoxin.
at 37 °C in an minimal essential medium (Grand Island Biological Co.), supplemented with 5% fetal calf serum by procedures described earlier (15, 19). Labeling of cells with [35S]methionine and [3H]leucine was carried out in growth medium lacking methionine and leucine, respectively, and supplemented with 10% fetal calf serum (minus methionine or leucine medium).

**Chemicals**—Taxol (NSC 125973) and maytansine (NSC 158586) were obtained from the Drug Synthesis and Chemistry Branch of the United States National Cancer Institute. Podophyllotoxin was purchased from Polysciences, Inc., Warrington, PA. Colchicine, colcemid, ouabain, and thioguanine (36) were used as described by Pipeleers et al. (20) and Cabral et al. (16), except that podophyllotoxin was replaced with 25 μM podophyllotoxin (wild type) line which has been selected for resistance to emetine (two steps), ouabain, and thioguanine (36). Colchicine, colcemid, and thioguanine were obtained from Sigma. Nocodazole was purchased from Aldrich. (+)-stegnacine was obtained from Aldrich. (±)-stegnacine was kindly provided by Dr. J. P. Robin (23) of Laboratoire de Synthese Organique, Le Mans, France.

**Measurement of Drug Resistance**—The degree of resistance of mutant cell lines towards various drugs was determined by seeding about 100 and 200 cells (in 0.5 ml of growth medium) into the wells of 24-well tissue culture dishes (Linbro, Falcon) containing 0.5 ml of the different dilutions of the drugs (including control, which contained no drug) made two times the final concentrations desired in the growth medium (15, 18). The dishes were incubated for 6–7 days at 37 °C, after which they were stained for about 30 min with 0.5% methylene blue in 50% methanol; subsequently the number of colonies was counted. The relative plating efficiencies (same cloning efficiencies) in the presence of different concentrations of the drugs were determined as the ratios of the number of colonies at a given drug concentration to that obtained in control cells plated in the absence of any drug.

**Labeling of Cells and Analysis of Proteins in Cell Extracts**—Cells were seeded into the wells of a 24-well tissue culture dish (Linbro, Falcon) at a concentration of about 1–2 × 105 cells/well. After 24–48 h, when cells had become nearly confluent, the dishes were removed and cells were rinsed with 2 ml of medium with methionine. The labeling of cells was carried out in 0.2 ml of medium minus methionine containing 50 μCi of [35S]methionine (specific activity, 1050 Ci/mmol; New England Nuclear). After 2 h of labeling at 37 °C, cells were rinsed with 2 ml of phosphate-buffered saline (containing, per liter, 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na2HPO4, and 0.085 g of KH2PO4) at room temperature, and then 100 μl of lysis buffer (5.6 μM urea, 1.6% Ampholine, pH 4–6, 0.4% Ampholine, pH 3–10, 5% β-mercaptoethanol, and 2% Nonidet P-40) was added. After allowing 10 min for lysis and solubilization, the cell lysate was centrifuged at 8000 × g for 3 min and then directly layered onto prerun isoelectric focusing gels.

**Two-dimensional Gel Electrophoresis**—The protocol employed was essentially that of O’Farrell (24) with minor variations. Isoelectric focusing gels contained 2.5% acrylamide, 4% Nonidet P-40, 4% Ampholines, pH 4–6, 1% Ampholines, pH 3–10, and 8.3 μM urea. N, N', N', N'-Tetramethyldiamine was not used during polymerization, and, instead, ammonium persulfate was used at a final concentration of 0.03%. The lysis buffer was used to overlay both the gels during polymerization and the samples during electrophoresis. The electrophoresis in the second dimension was carried out in 10% polyacrylamide gels essentially as described by O’Farrell (24), and the gels were run for 16 h at 90 V. The slab gels were dried with or without pretreatment with an autoradiographic enhancer (ENHANCE, New England Nuclear), exposed to X-Omat XR-1 films (Kodak), and then processed by an automatic x-ray film processor.

**One-dimensional Peptide Mapping**—The analysis of peptides obtained after limited proteolysis was carried out as described by Cleveland et al. (25). The protein spots for peptide mapping were excised from the dried two-dimensional gels. The exact positions of protein spots after excision were determined by autoradiograms (gels premarked with radioactive ink for proper alignment). The excised gel pieces containing the protein were soaked in a buffer containing 0.125 M Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate and 1 mM EDTA for 30 min and then pushed into the wells of a second sodium dodecyl sulfate-acrylamide gel (separating, 10% separating). The gel slices were overlaid with the above buffer containing 10% glycerol and 0.2 μg of Staphylococcus V8 protease (Miles) and 0.001% of bromphenol blue. The gels were electrophoresed for 120 V until the bromphenol blue dye had nearly reached the bottom of the stacking gel, at which time the current was turned off for 30 min to allow digestion. The gels were subsequently electrophoresed in the normal manner until the tracking dye had reached the bottom of the separating gel. The gels were then fixed, treated with the autoradiographic enhancer, dried, and exposed to x-ray films.

**Analysis of Microtubule-associated Proteins**—Two methods were employed for the analysis of microtubule-associated proteins. In the method of Cabral et al. (16), assembled microtubules were separated from free tubulin and other soluble proteins by lysing cells in a microtubule-stabilizing buffer. For such studies, cells growing in 60-mm dishes were labeled with [35S]methionine for 2 h and then washed twice with phosphate-buffered saline. Cells were lysed by the addition of 3 ml of a microtubule-stabilizing buffer (50% glycerol, 5% dimethyl sulfoxide, 0.5 mM GTP, 0.5 mM MgCl2, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-N,N',N',N'-tetraacetic acid, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 10 mM potassium phosphate buffer, pH 6.95), whose composition was similar to that described by Pipeleers et al. (20) and Cabral et al. (16), except that phenylmethylsulfonyl fluoride was substituted for the protease inhibitor aprotinin. After about 5 min, cells were scraped from the dishes using a rubber policeman and transferred to centrifuge tubes. The tubes were vortexed vigorously for about 10 s and then centrifuged at 100,000 × g for 60 min at 25 °C to obtain a microtubule-rich fraction. The microtubule-rich pellet thus obtained was extracted in the cold with about 2.5 ml of a cold buffer containing 10 mM Tris-HCl, pH 6.8, and 0.1% Triton X-100. The cold extraction dissociates microtubules, and the various constituent proteins are obtained in the supernatant when a second centrifugation at 100,000 × g for 60 min at 0–4 °C is carried out. These proteins, after precipitation with 80% acetone, were dissolved in the lysis buffer and then applied onto isoelectric-focusing gels.

In the second method, detergent extraction of cells was carried out as described by Solomon et al. (26) and Duerr et al. (27). In these studies, cells growing on 60-mm dishes were labeled with [35S]methionine (200 μCi/dish) for 4 h. To one set of dishes, 5 × 106 M colchicine was added during the final 2 h of labeling. Following labeling, cells were rinsed twice with phosphate-buffered saline and then washed once with the extraction buffer (0.1 M 1,4-piperazineethanesulfonic acid, 1 × 10−3 M MgSO4, 2 × 10−3 M ethylene glycol bis(β-aminoethyl ether)-N,N',N',N'-tetraacetic acid, 1 × 10−3 M GTP, and 2 μM glycine, pH 6.9). The washed cells were treated with the extraction buffer containing 0.1% Nonidet P-40 for 15 min to lyse the cells. The supernatant was removed and the cytoskeletons which remained associated with the dishes were rinsed twice with the extraction buffer. The cytoskeletons thus obtained were then treated with the extraction buffer containing 5 mM CaCl2 for 15 min, and the proteins released in this buffer were precipitated with acetone and analyzed by two-dimensional gel electrophoresis.

**RESULTS**

The selection of CHO mutant cell lines which are resistant to podophyllotoxin has been described earlier (18). The dose-response curves of some of these cell lines towards podophyllotoxin are presented in Fig. 1. The PodRmut class of mutants, which show a higher level of resistance to podophyllotoxin than the PodRmut mutants, were obtained after a second step selection on PodRmut 16 cells. The PodRmut and PodRmut mutants showed a very similar degree of resistance towards podophyllotoxin (Fig. 1).

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>CHO cell lines used in the present studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>Phenotype and origin</td>
</tr>
<tr>
<td>Pro (wild type)</td>
<td>Proline-requiring CHO cell line used for various mutant selections (15-18)</td>
</tr>
<tr>
<td>EOT</td>
<td>Pro (E. O. T.) OuA&quot; Thg&quot;</td>
</tr>
<tr>
<td>PodR8 and PodR16</td>
<td>Two steps podophyllotoxin-resistant mutants selected from EOT cells (18)</td>
</tr>
<tr>
<td>PodR2</td>
<td>Two steps podophyllotoxin-resistant mutants selected from PodR16 cells (18)</td>
</tr>
<tr>
<td>PodR3</td>
<td>Two steps podophyllotoxin-resistant mutants selected from PodR16 cells (18)</td>
</tr>
<tr>
<td>PodR6</td>
<td>Two steps podophyllotoxin-resistant mutants selected from PodR16 cells (18)</td>
</tr>
<tr>
<td>PodR17</td>
<td>Two steps podophyllotoxin-resistant mutants selected from PodR16 cells (18)</td>
</tr>
<tr>
<td>PodR18 and PodR18</td>
<td>Two steps podophyllotoxin-resistant mutants selected from PodR16 cells (18)</td>
</tr>
<tr>
<td>Hyb Pro × PodR16-2</td>
<td>Two independent hybrid clones picked from a cross between Pro and PodR16 lines (18)</td>
</tr>
<tr>
<td>Hyb Pro × PodR16-4</td>
<td>Two independent hybrid clones picked from a cross between Pro and PodR16 lines (18)</td>
</tr>
</tbody>
</table>
In order to characterize these mutants, we first examined their cross-resistance patterns towards various other inhibitors of microtubule assembly, such as colchicine, vinblastine, griseofulvin, nocodazole, stegnacine, taxol, and maytansine. In addition, the cross-resistances of mutant cells towards VM-26 (which is a podophyllotoxin analog which does not inhibit microtubule assembly (28)), and to puromycin (to which many permeability mutants show cross-resistance) were also determined. The experiments were carried out by determining the plating efficiencies of two PodR' mutants and four PodR" mutants in the presence of different concentrations of the above drugs. (The cross-resistance of a few of these mutants towards colchicine, colcemid, and vinblastine was reported earlier using a semiquantitative procedure (18). However, in view of some ambiguity involved in recording the results of such experiments and the fact that old drug stocks were used in such studies, the cross-resistance studies with the above drugs were repeated.)

The results of cross-resistance studies are shown in Fig. 2. The two PodR' mutants (PodR'8 and PodR'6) which were examined both showed increased resistance towards colchicine, colcemid, and stegnacine, and a very slight cross-resistance towards nocodazole (Fig. 2, A, B, D, and G). At the same time, both of these PodR' mutants became highly sensitive to taxol and showed a slightly higher sensitivity towards maytansine. The level of resistance of these mutants towards other microtubule inhibitors, such as vinblastine and griseofulvin, or to other compounds, such as VM-26 and puromycin (results not shown), however, remained unchanged.

In going from the first step of podophyllotoxin resistance to the second step (i.e. from PodR' to PodR"), the cross-resistance pattern of mutant cell lines towards various microtubule inhibitors again showed large and interesting changes. However, unlike the two PodR' mutants, which showed very similar patterns of cross-resistance, the PodR" mutants appeared to be of two distinct kinds based on their cross-resistance patterns. One type of PodR" mutant, which includes PodR"2, PodR"6, and PodR"7 (results for PodR"7 not shown, since it behaves very similarly to PodR"6), showed increased resistance towards nocodazole, vinblastine, maytansine, and taxol, while at the same time becoming more sensitive to colchicine, colcemid, and stegnacine, in comparison to the parental PodR'16 cells. The sensitivity of this group of PodR" mutants towards colchicine is, in fact, even higher than that of the parental sensitive cell line (Fig. 2A). However, these mutants did not show any increased resistance towards griseofulvin (Fig. 2E), VM-26 (Fig. 2C), or puromycin (results not shown). The PodR"3 cell line comprises the other type of PodR" mutant. This mutant cell line, in comparison to the PodR'16 cells, showed increased resistance towards all of the inhibitors of microtubule assembly but did not show any cross-resistance to the podophyllotoxin analog VM-26 or to puromycin.

Based on their cross-resistance patterns, the PodR' and PodR" mutants which we have obtained are distinct from the colchicine-, colcemid-, and griseofulvin-resistant mutants which have been described earlier (16, 21, 22). The lack of cross-resistance of these mutants to the podophyllotoxin an-
Fig. 3. Two-dimensional gel electrophoretic patterns of proteins from different cell types. A, EOT line (PodS); B, PodR16; C, PodR16; D, PodR17. The positions of molecular weight markers as obtained after running a parallel gel are shown in C. The molecular weight markers employed were phosphorylase b ($M_r = 94,000$), albumin ($M_r = 67,000$), ovalbumin ($M_r = 43,000$), and carbonic anhydrase ($M_r = 30,000$). The arrows indicate the positions of tubulin (T), the P protein (P) and the mutant (M) form of the P protein.
alog VM-26 and to puromycin also indicates that the lesions in these mutants do not involve a general permeability alteration.

**Gel Electrophoresis Analysis of the Pod\(^{R}\) Mutant Cell Lines**—To find out the possible biochemical alteration that may have occurred in the Pod\(^{R}\) mutants, the patterns of proteins present in the sensitive and mutant cell lines were compared by two-dimensional gel electrophoresis. The gel electrophoretic patterns of proteins from a number of sensitive and resistant cell lines are shown in Fig. 3. The two Pod\(^{R}\) mutants which were examined did not show any apparent change in the electrophoretic mobility of tubulins or any other protein (see Fig. 3B for Pod\(^{R}\)16; results for Pod\(^{R}\)8 not shown). The Pod\(^{R}\) mutants which were examined again did not show any clear and consistent change in the tubulin region. However, in two of the Pod\(^{R}\) mutants, Pod\(^{R}\)6 and Pod\(^{R}\)7, a new spot (labeled M) was seen in all experiments. Pod\(^{R}\)6 and Pod\(^{R}\)7 have been independently obtained from Pod\(^{R}\)16 in separate experiments. However, in view of their identical pattern of cross-resistance to various microtubule inhibitors (Figs. 1 and 2), it is possible that these mutants may bear identical genetic lesions. This new spot was not seen in the other Pod\(^{R}\) mutants, e.g. Pod\(^{R}\)2 and Pod\(^{R}\)3, which were examined (results not shown). Based on an experiment where standard molecular weight markers were run in a parallel gel (positions shown in Fig. 3C), the molecular weight of this new protein was estimated to be approximately 66,000.

The position of spot M in Pod\(^{R}\)6 and Pod\(^{R}\)7 indicated that it may have arisen by a charge alteration in the adjacent protein (labeled P in Fig. 3, C and D). To find out whether it was indeed so, the P spot from the sensitive cells and both the P and M spots from the Pod\(^{R}\)6 cells were excised, and the proteins in these spots were analyzed by means of one-dimensional gel electrophoresis after partial digestion with *Staphylococcus* V8 protease (25). Results of these studies are shown in Fig. 4. As can be seen, the peptide fragments which are obtained after partial digestion of protein P (lanes A and B, from the wild type and Pod\(^{R}\)6 cells, respectively) are very similar to those obtained from spot M (lane C). The only difference between the P and the M peptides is the presence of a high molecular weight peptide in M (shown by arrow in lane C) which is absent in P. Instead, the P protein contains one or two peptides in the low molecular weight range (shown by arrows in lane B) which are not present in the M protein. The above results would be expected if the mutation leading to the appearance of the M spot (from P) changes a protease-sensitive site into an insensitive one. Lanes D and E in Fig. 4 show the tryptic peptides which are obtained after the partial protease digestion of the α-tubulin spots (of the two tubulin spots, the one which has higher molecular weight) from the wild type and Pod\(^{R}\)6 cells, respectively. The peptide fragments from tubulin are distinct from those obtained from either the P or the M spots. These results strongly suggest that the new protein spot M in the mutant cells is derived from the protein spot P by a mutational alteration.

The above inference is also supported by an independent approach which involves analyses of these proteins in somatic cell hybrids. We have earlier shown that cell hybrids formed between Pod\(^{R}\) and Pod\(^{S}\) cells show intermediate levels of resistance to podophyllotoxin, indicating a co-dominant nature of the Pod\(^{R}\) lesion (18). Accordingly, the presence of a new protein spot in the mutant cells suggests that only one of the two gene copies for this protein has been altered such that the parental cells contained only the wild type form of this protein, whereas in the mutant cell, both the wild type and the altered forms were present. If this view was correct, then cell hybrids formed between mutant and sensitive cells, where the sensitive to resistant gene ratio will be 3:1, should contain an excess of the wild type (P) form of this protein as compared to the mutated (M) form. To test this prediction, two-dimensional gel electrophoresis of several Pod\(^{R}\) × Pod\(^{S}\) hybrid clones was carried out, and the results for two representative hybrid clones are shown in Fig. 5. As can be seen, the mutant form of protein was again clearly seen in both the hybrid clones; however, as expected, its amount in comparison to the P form was markedly diminished in both of the clones. Denitomietic quantitation of the P and M spots in several different experiments (results not shown) showed that their relative ratio in Pod\(^{R}\)6 and Pod\(^{R}\)7 cells was about 1.0:8. In contrast to mutant cells, the ratio of these two spots in hybrid clones was only about 10:25. These results support the view that the new spot M seen in mutant cells most likely represents an altered form of the protein P.

**Analysis of Microtubule-associated Proteins**—Podophyllotoxin is considered to be a highly specific inhibitor of micro-
tubule assembly in eukaryotic cells (2, 3, 9, 35). In view of this, the alteration seen in our mutants in a protein distinct from tubulin was unexpected. To determine whether this protein may represent one of the microtubule-associated protein, two types of experiments were carried out.

In one approach, the assembled microtubules were separated from the majority of free tubulin and other soluble proteins by cell lysis in a microtubule-stabilizing buffer and then centrifuging the extract at 25 °C. When the microtubule fraction thus obtained (i.e. pellet) is extracted in cold, microtubules are dissociated, and upon subsequent centrifugation at 4 °C, tubulin and other microtubule-related proteins are released in the supernatant. The proteins which are found in the cold supernatants of wild type and PodRU6 cells were analyzed by two-dimensional gel electrophoresis. Results of these studies are shown in Fig. 6. As can be seen in Fig. 6A, the proteins thus obtained from wild type cell contained only a few spots in addition to tubulins and actin. However, a spot corresponding to the protein P was clearly present in the wild type cells (as one of the major spots), suggesting that this protein may represent a microtubule-associated protein. More interestingly, when the proteins from PodRU6 cells were similarly analyzed, both the P and the M spots were observed, in addition to tubulin and some other proteins. These results suggest that the protein P (and its mutant form M) most likely represents a microtubule-related protein.

The second approach that we have used to show that the protein P is most likely a microtubule structural component is based upon the work of Solomon et al. (26) and Duerr et al. (27). In this approach, cytoskeletons are prepared from cells under conditions which preserve the original microtubules of the cells. When cytoskeletons thus prepared are extracted with buffers containing $5 \times 10^{-3}$ M Ca$^{2+}$ (which depolymerize microtubules), various microtubule-associated proteins are released. The microtubules specificity of any protein is shown by preparing cytoskeletons under conditions where intact microtubules are not present in cells (e.g. colchicine pretreatment) and then carrying out extraction with the Ca$^{2+}$-containing buffer. In this approach, when a protein is released from only those cytoskeletons in which microtubules are preserved, then it represents a microtubule-associated protein. Following this approach, we have analyzed the proteins which are released by Ca$^{2+}$ from cytoskeletons prepared from wild type and PodRU6 cells (both control and colchicine-pretreated). The results of these experiments are shown in Fig. 7. As seen in Fig. 7A, when cytoskeletons prepared from wild type cells are treated with Ca$^{2+}$, four major spots in addition to the actin and tubulin are seen, and the protein spot corresponding to P is clearly a prominent spot under these conditions. In the case of PodRU6 cells (Fig. 7C), the protein spots corresponding to both P and M are seen under these conditions. When the cytoskeletons prepared from cells pretreated with colchicine were similarly extracted with Ca$^{2+}$, only the spot corresponding to actin was seen (Fig. 7B), and the other major protein spots seen in Fig. 7, A and C, were not present. These results suggest that the protein P (and its mutant form M) as well as a number of other proteins, labeled 1, 2, and 3 in Fig. 7, A and C, probably represent microtubule-associated proteins.

The gel patterns shown in Figs. 3 and 5–7 indicated that the 66,000-dalton protein which is affected in the mutant cells was present in the parental cells in amounts comparable to

![Fig. 5. Gel electrophoretic patterns of proteins from two PodRU6 × Pro- (wild type) hybrid clones. A, hybrid Pro × PodRU6-2; B, hybrid Pro × PodRU6-4.](http://www.jbc.org/)

![Fig. 6. Two-dimensional gel electrophoretic patterns which are released from assembled microtubules upon cold extraction. The experiment was carried out as described under “Experimental Procedures.” (Only the tubulin and nearby regions of the gels are shown.) A, Pro- cells; B, PodRU6 cells.](http://www.jbc.org/)
that seen for tubulins. However, in view of the fact that labeling of cells in these experiments was carried out with \[^{35}S\]methionine, whose contents could vary greatly among different proteins, further confirmation of this result was necessary. To obtain more accurate estimate of the relative amounts of these proteins, cells were labeled with \[^{3}H\]leucine for 20 h, and then analyzed by two-dimensional gel electrophoresis. The gel patterns shown in Fig. 8 were obtained after exposing gels for relatively short periods to identify only the major cellular proteins. As can be seen, the autoradiograms from both the parental and mutant cells contained a number of proteins, including actin, tubulins, and the P protein. The mutant cells also contained the M spot in amounts similar to that seen for the P protein. Densitometric quantitation of the relative intensities of the P and M protein spots in these gels in comparison to the tubulin spots indicates that the ratio of the P protein (or P + M in case of the mutant) to tubulin in cell extracts is approximately 1.3:1, which is very close to the ratio of molecular weights of these proteins (1.22:1; for tubulin, an average molecular weight of 54,000 has been assumed). These results indicate that the 66,000-dalton protein is present in cells in nearly the same molar amount as tubulins.

DISCUSSION

Results presented in this paper show that the genetic lesions in two independently isolated second step mutants resistant to the microtubule inhibitor podophyllotoxin (Pod\(^{II}\)) affect a protein with an approximate molecular weight of 66,000 (designated P, for podophyllotoxin resistance). Podophyllotoxin is a very specific inhibitor of microtubule assembly, both in \textit{vivo} and in \textit{vitro}, and hence mutants resistant to it (which, based on the cross-resistance and other studies presented here, are not affected in membrane permeability) should affect a component involved in microtubule function and/or assembly. Two kinds of experiments which are described in this paper provide strong indications that the protein affected in these mutants constitute a microtubule-associated protein. Using one of these approaches for the analysis of microtubule-associated proteins, Solomon \textit{et al.} (26) and Duerr \textit{et al.} (27) have recently identified a protein with a molecular weight of about 69,000 which was present in the cells of all rodent species examined, including hamster. It is possible that the protein P which is affected in our mutants may correspond to this protein.

Another important observation that has emerged from our studies is the relative amount of the P protein as compared to tubulin. The data presented here show that the P protein is present in cell extracts in nearly the same molar ratio as tubulin. If the P protein is indeed a microtubule-associated protein, as has been indicated by our studies, then in view of the above observation, it would comprise a major microtubule structural component.

It should be mentioned that the identification of a protein as a microtubule-associated protein, in the past, has generally been based on the criteria that such proteins co-assemble with microtubules when repeated polymerization/denpolymerization of microtubules is carried out \textit{in vitro}. However, this approach of identifying microtubule-associated proteins has several limitations. The most important of these is that \textit{in vitro} polymerization/denpolymerization of microtubules in general is carried out under conditions (in the presence of 1-6 M glycerol) which may not require the presence of many microtubule-associated proteins. The nature of proteins which are found associated with microtubules has also been found to vary in such studies, depending upon the polymerization/denpolymerization conditions which are employed (5-8, 29-32). For example, Weatherbee \textit{et al.} (6) have reported that when microtubule assembly was carried out from HeLa cell extracts in the absence of glycerol, a protein with a molecular weight of 68,000 was the major non-tubulin protein that assembled under these conditions. However, this protein was virtually absent when polymerization was carried out in the presence of glycerol. In the above approach, the specificity of the microtubule-associated proteins is determined by their ability to stimulate microtubule assembly \textit{in vitro}. This is based on the assumption that all microtubule-associated proteins are required for the assembly process, which may not be true. It is quite possible that some of the microtubule-associated proteins may be specifically bound to assembled microtubules (assembled to the stage as studied \textit{in vitro}). For example, the 68,000-dalton protein identified by Weatherbee \textit{et al.} (6) does not stimulate tubulin polymerization \textit{in vitro}, although this protein does specifically interact with the polymerized microtubules (33). In a few experiments that we have carried out on the copolymerization of proteins from CHO cell extracts with calf brain tubulin (in the presence of glycerol), the P protein has been found not to co-assemble with microtubules. However, in view of the above-mentioned limitations of this approach, these experiments are inconclusive, and further investigation of the microtubule assembly process using many different conditions need to be carried out.

Podophyllotoxin is known to be a competitive inhibitor of colchicine binding to tubulin and is believed to bind to the same site as colchicine (2, 9, 34, 35). In this regard, our finding that mutants resistant to podophyllotoxin are affected in a protein other than tubulin is unexpected. However, the cross-resistance studies with the mutant cells which show that, whereas Pod\(^{II}\) mutants exhibit increased resistance to colchicine, some of the Pod\(^{III}\) mutants become highly sensitive to this drug, providing a strong indication that although the sites of action of these two drugs interact with each other, they are not identical. One possible way to explain the competitive effect of podophyllotoxin on colchicine binding would be if the protein P exists as a complex in cells in association with tubulin and induces in it a conformational change that may be necessary for colchicine binding. In such a case, the binding of podophyllotoxin to protein P may cause its dissociation from tubulin, which would result in loss of colchicine-binding ability of the latter and give the appearance of competitive inhibition. The complex of protein P and tubulin may in fact be somewhat unstable under the normal \textit{in vitro} conditions, thus accounting for the gradual loss of colchicine-binding ability of various cell extracts (9).

The cross-resistance of various PodR mutants to other inhibitors of microtubule assembly provides valuable information regarding the site of action of these drugs and the nature of mutations in such cells. For example, the increased resistance of PodR mutants to colchicine, colcemid, nocodazole, and stegnacine, and their collateral sensitivity to taxol, indicate that the mutation in such cells affects the binding sites of all these drugs, but not that of griseofulvin and vinblastine (to which the mutant cells do not exhibit cross-resistance or sensitivity). Based on their cross-resistance patterns, the PodR and PodRIT classes of mutants that we have thus far obtained each appear to be of more than one kind (these studies and Footnote 2). Further biochemical studies with these mutants should provide valuable information regarding the mechanisms of action of various microtubule inhibitors and regarding the structure and functions of microtubules in mammalian cells.

Acknowledgments—We thank Drs. H. P. Ghosh and K. B. Freeman for helpful comments on the manuscript.

REFERENCES

Podophyllotoxin-resistant mutants of Chinese hamster ovary cells. Alteration in a microtubule-associated protein.
R S Gupta, T K Ho, M R Moffat and R Gupta