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The Characterization of Plasma Membrane-Bound Tubulin of Cauliflower Using Triton X-114 Fractionation[1]

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The cortical microtubules determine how cellulose microfibrils are deposited in the plant cell wall and are thus important for the control of cell expansion. To understand how microtubules can control microfibril deposition, the components that link the microtubules to the plasma membrane (PM) of plant cells must be isolated. To obtain information on the properties of the tubulin-membrane associations, cauliflower (*Brassica oleracea*) PM was subjected to Triton X-114 fractionation, and the distribution of α- and β-tubulin was analyzed using immunoblotting. Approximately one-half of the PM-associated tubulin was solubilized by Triton X-114 and 10 to 15% of both α- and β-tubulin was recovered in the detergent phase (indicative of hydrophobic properties) and 30 to 40% was recovered in the aqueous phase. The hydrophobic tubulin could be released from the membrane by high pH extraction with preserved hydrophobicity. A large part of the PM-associated tubulin was found in the Triton-insoluble fraction. When this insoluble material was extracted a second time, a substantial amount of hydrophobic tubulin was released if the salt concentration was increased, suggesting that the hydrophobic tubulin was linked to a high-salt-sensitive protein aggregate that probably includes other components of the cytoskeleton. The hydrophobicity of a fraction of PM-associated tubulin could reflect a direct or indirect interaction of this tubulin with the lipid bilayer or with an integral membrane protein and may represent the anchoring of the cortical microtubules to the PM, a key element in the regulation of cell expansion.

In plant cells MTs appear in a variety of highly ordered arrays: the cortical array, the preprophase band, the spindle, and the phragmoplast (Lloyd, 1987; Lambert, 1993; Cyr and Palevitz, 1995). Interphase cells are characterized by cortical MTs aligned in roughly parallel arrays under the PM. The orientation of the cortical MTs is under strict developmental control via several signals including hormones, light, and gravity (Nick et al., 1990; Blancaflor and Hasenstein, 1993; Zandomeni and Schopfer, 1993; Shibaoka, 1994). It has been proposed that the cortical MTs influence the deposition of newly synthesized cellulose microfibrils in the cell wall, thereby determining the direction of cell expansion (for reviews, see Gunning and Hardham, 1982; Giddings and Staehelin, 1991).

To understand the dynamics of the cortical MTs and their control over cellulose-microfibril deposition, the link between the PM and the MTs needs to be identified. There are several examples of MAPs associated with cortical MTs, but so far it is not known whether they constitute the actual link between MTs and the PM. Examples of plant MAPs include a 76-kD MAP of carrot (*Daucus carota*) suspension cells (Cyr and Palevitz, 1989), an 83-kD maize protein immunologically related to τ (Vantard et al., 1991), the elongating factor 1-α in carrot suspension cells (Durso and Cyr, 1994), and a 65-kD MAP of tobacco (*Nicotiana tabacum*) BY-2 cells (Chang-Jie and Sonobe, 1993).

Plant cells lack centrosomes, which are the structures that constitute MT-organizing centers in animal cells. In plant cells, MTs are organized at different surfaces depending on the cell cycle, such as at the PM in interphase and preprophase, and at the nuclear membrane at the onset of mitosis. Proteins similar to MT-organizing-center proteins also seem to be present in plants. Liu et al. (1994) showed the presence of γ-tubulin, although in plants this protein is distributed all along the MTs (excluding the plus ends). A monoclonal antibody against animal centrosomal material, 6C6, has been shown to recognize a 77-kD pollen tube protein (Cai et al., 1996). A 120-kD MAP has been isolated from detergent-extracted carrot protoplast cytoskeletons, and although antibodies against this protein labeled all MT arrays, the cortical MTs were labeled in a more punctuate manner, suggesting that the 120-kD polypeptide was involved in a PM-MT interaction or in a MT nucleation (Chan et al., 1996).

The interaction between tubulin and the PM has also been studied with isolated membranes: a 50-kD MAP expressed after red-light irradiation of elongating maize coleoptiles was copurified with the PM (Nick et al., 1995). The above-mentioned, possibly centrosome-related, 77-kD protein was enriched in an isolated PM fraction (Cai et al., 1996). A 90-kD MAP was isolated from a 3-[(cholamido propyl)dimethyl-ammonio]-1-propanesulfonic acid-solubilized membrane fraction of tobacco BY-2 cells (Marc et al., 1996). In studies on tobacco leaves, the resistance of PM-associated tubulin to extraction in high and low ionic strength and in the nonionic detergent Nonidet P-40 was interpreted as tubulin behaving

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Abbreviations: ICM, intracellular membrane; MAP, microtubule-associated protein; MT, microtubule; PM, plasma membrane.

like an integral membrane protein. The hypothesis put forward was that this tubulin could constitute the actual link between the cortical MTs and the PM (Laporte et al., 1993).

Triton X-114 fractionation is frequently used to determine whether membrane proteins are integral or peripheral (Bordier, 1981). In a study of rat brain membranes, roughly one-third of the membrane-associated α-tubulin (β-tubulin was not assayed) was recovered in the detergent fraction, i.e. the α-tubulin behaved as an integral membrane protein (Beltramo et al., 1994). However, α-tubulin could be released from the membranes by high pH, and Triton X-114 fractionation of the released proteins led to the recovery of α-tubulin in the aqueous phase only. It was concluded that the characteristic hydrophobic nature of α-tubulin in the membrane fraction came about through an interaction between α-tubulin and a membrane component, an interaction that was broken by high pH treatment (Beltramo et al., 1994).

In the present work well-characterized PM (Sonesson and Widell, 1993), ICM, and cytosol of cauliflower (*Brassica oleracea*) inflorescences were subjected to Triton X-114 extractions and analyzed with regard to the distribution of α- and β-tubulin. It was found that with plant membranes there is a pool of tubulin that displays hydrophobic behavior similar to that of rat brain membranes. This tubulin could be released from the membrane by high pH, without the loss of its hydrophobicity.

MATERIALS AND METHODS

The outermost layer (2–3 mm) of cauliflower (*Brassica oleracea*) inflorescences was used. This tissue consists of differentiating cells and thus has relatively low concentrations of proteases and Ca^{2+} compared with other parts of the inflorescence.

PM Isolation

PMs were purified by aqueous polymer two-phase partitioning, as described by Sonesson and Widell (1993), with some changes: the centrifuge fraction used was 1,000 to 20,000*g* instead of 1,000 to 10,000*g*; after the initial phase separation, the upper phase (enriched in PM) was washed with four, fresh, lower phases, and the lower phase (ICM) was washed with four, fresh, upper phases. The final PM fraction was the equivalent of fraction six described by Sonesson and Widell (1993), whereas the ICM fraction was the equivalent to fraction one described in the same paper. After ultracentrifugation (100,000*g*), the membranes were resuspended in control medium: 20 mM Hepes-NaOH, pH 7.4, 138 mM NaCl, 6 mM KCl, 5 mM $NaHCO_3$, 1 mM Na_2HPO_4, 2 mM DTT, and 1 mM EDTA. The supernatant after the 20,000*g* centrifugation was further centrifuged at 300,000*g* for 1 h to yield the cytosolic fraction. Samples were frozen in liquid N_2 and stored at −80°C until use.

Inside-out (cytoplasmic side-out) PM vesicles were obtained according to the method of Johansson et al. (1995) by the addition of Brij 58 (Sigma) to 0.05% (w/v) at a detergent:protein ratio of 10:1. After 30 min on ice, the inside-out PM vesicles were collected at 100,000*g* for 1 h and resuspended in control medium. All preparation procedures were performed at 4°C.

Triton X-114 Extraction

Triton X-114 (Sigma) was precondensed according to the method of Bordier (1981). The extraction procedure was that of Beltramo et al. (1994). To samples of 1 mg mL^{-1} (or less; see figure legends) in control medium, Triton X-114 was added at 0°C to 0.5% (w/v). After thorough mixing and incubation for 60 min at 0°C, the detergent-insoluble material was collected at 180,000*g* for 45 min. The supernatant was incubated in a water bath at 35°C for 3 min and then centrifuged at 1,000*g* for 3 min to obtain phase separation. The aqueous upper phase and the detergent-rich lower phase were separated and cooled before the addition of Triton X-114 and control medium, respectively, so that the concentration of Triton X-114 was again 0.5%. These mixtures were incubated at 0°C for 15 min before incubation at 35°C and centrifugation as described above. This washing procedure was repeated.

When indicated, the detergent-insoluble material was resuspended in control medium containing 0.7 M NaCl and reextracted with Triton X-114 as described above.

High-pH Extraction of PM

High-pH extraction was performed to strip the PM vesicles of tubulin. Solutions with pH values of 9.0, 10.0, 11.0, and 11.4 were obtained by mixing 0.1 M Na_2CO_3 with 0.1 M $NaHCO_3$. A pH 12.0 solution was obtained by adding 0.2 M NaOH (4 mL) to 0.1 M Na_2CO_3 (40 mL). Concentrated samples of right-side-out or inside-out PM were diluted in the respective or in control CO_3^- solution medium and incubated on ice for 30 min. The vesicles were pelleted at 180,000*g*, resuspended in control medium, and fractionated with Triton X-114 as described above.

Protein

Protein was measured as described by Bearden (1978), with BSA as standard.

Electrophoresis and Immunoblotting

Proteins were separated by SDS-PAGE (Laemmli, 1970) on 10% polyacrylamide slab gels. Samples were solubilized 1:1 in a cocktail containing 0.125 M Tris-HCl, pH 6.8, 10% (w/v) SDS (the high SDS concentration was used to avoid Triton X-114-induced disturbances in protein migration), 20% (v/v) glycerol, and 100 mM DTT and boiled for 3 min before application to the gel. For fractions in which the amount of tubulin was suspected to be low, especially the detergent phases, proportionally more than of the other phases were applied to the gels. Except for fractions containing a high concentration of salt, the aqueous phases were concentrated (SpeedVac, Savant Instruments, Inc., Farmingdale, NY) to one-fourth of their original volume. Volumes applied to the gels are described in the figure legends as percentages of the total volume.

Electrotransfer of the separated proteins to PVDF Immobilon membranes (Millipore) was performed using a wet-blot apparatus (Bio-Rad) with 0.025 M Tris, 0.192 M Gly, 20% (v/v) methanol, and 0.0375% (w/v) SDS as a transfer buffer. Membranes were blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% [w/v] Tween 20) containing 2% (w/v) BSA for 60 min.

Primary antibodies were monoclonal anti-α-tubulin (N 356) diluted 1:500 and monoclonal anti-β-tubulin (N 357) diluted 1:500 (Amersham). As a secondary antibody, horseradish peroxidase-conjugated anti-mouse IgG (Amersham) diluted 1:10,000 was used. The enhanced chemiluminescence method (RPN 2108, Amersham) was used to detect the antigen.

Quantification of α-/β-Tubulin on Immunoblots

To assist in the interpretation of the distribution of tubulin (see Figs. 1 and 3), the relative intensities of the bands were determined and used to calculate the recovery. The enhanced chemiluminescence films were scanned in a OneScanner using Ofoto 2.0 software (Apple Computer Inc., Cupertino, CA), and the relative amounts of antigen were determined with scanning software (ScanAnalysis, BioSoft, Cambridge, UK).

RESULTS

Distribution of Tubulin after Triton X-114 Fractionation of PM, ICM, and Cytosol Fraction

Triton X-114 fractionation resulted in three phases: a Triton-insoluble pellet (collected at 180,000*g*), a detergent phase, and an aqueous phase. Roughly one-half of the recovered α- and β-tubulin in the cytosolic fraction and in the PM was found in the Triton-insoluble pellet (Fig. 1). In the ICM fraction a large portion of the recovered tubulin was also found in the detergent-insoluble pellet (note the considerably lower specific abundance of tubulin in the ICM fraction in Fig. 1). After phase-separation of the Triton-soluble PM proteins, α- and β-tubulin were found in both the detergent phase and in the aqueous phase. The recovery of α-tubulin was 12% in the detergent phase compared with 38% in the aqueous phase, and for β-tubulin, 16 and 33%, respectively (Fig. 1). In contrast, after fractionation of the cytosolic fraction, tubulin was found only in the aqueous phase. For the ICM, β-tubulin but not α-tubulin was recovered in both the detergent phase and in the aqueous phase (Fig. 1).

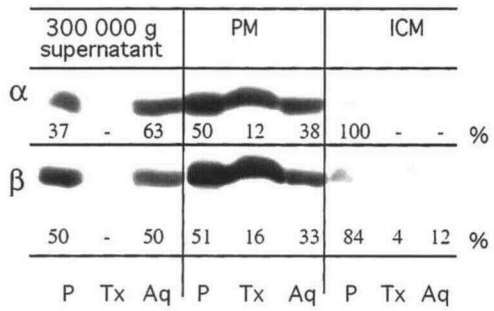

Figure 1. Immunoblots showing the presence of α- and β-tubulin in fractions obtained by Triton X-114 extraction of the 300,000*g* supernatant, PM, and ICM. Samples at 1 mg mL^{-1} were incubated with Triton X-114 (0.5% [w/v] final concentration) at 0°C for 60 min. The detergent-insoluble material (P) was collected at 180,000*g* and the soluble material was partitioned into a detergent phase (Tx) and an aqueous phase (Aq) by heating the sample to 35°C. Aliquots of 4% of the detergent-insoluble fraction (P), 20% of the detergent fraction (Tx), and 4% of the aqueous fraction (Aq) were subjected to SDS-PAGE and immunoblotting. The numbers in the figure represent the percentage of total α- or β-tubulin recovered after each Triton X-114 extraction, as determined by scanning software.

The number of times the Triton phases were washed had little effect on the amount of tubulin found in the detergent phases (not shown), demonstrating that the presence of hydrophobic tubulin was not merely an effect of tubulin equilibrating between the two phases. Rather, these results indicate that in plant membranes a fraction of the co-purified tubulin had hydrophobic properties, in agreement with the results of a study of brain membranes by Beltramo and co-workers (1994).

To rule out the possibility that tubulin was collected in the detergent phase because of a heat-stimulated protein assembly, fractionation was also done in the presence of 0.5 M Suc. In such a system the detergent phase is formed on top of the aqueous phase instead of below, and precipitated proteins still pellet. Tubulin was recovered in the detergent phase, regardless of whether Suc was present. An extended incubation time at 37°C (to 1 h) also had no effect on the amount of hydrophobic tubulin (not shown). Hydrophobic tubulin was also found when fresh membranes were used (not shown).

Triton X-114 Fractionation after High-pH Extraction of PM

The hydrophobic behavior of tubulin is probably attributable to its interaction with the PM. It was therefore interesting to see how the release of tubulin (from the PM) affected this hydrophobicity. Other findings were that both α- and β-tubulin decreased and finally disappeared from the PM pellet after treating isolated cauliflower PM with 0.1 M CO_3^- medium at increasing pH (A. Sonesson and S. Widell, unpublished data). Therefore, PM washed in media with a range of pH values were subjected to Triton fractionation (Fig. 2). After washing at pH 11.0 or higher, no hydrophobic α- or β-tubulin could be found in the PM pellet. More hydrophobic tubulin was found with untreated PM than with Brij 58-treated PM, probably because there was a general loss of protein with the Brij 58 treatment.

The absence of hydrophobic tubulin in the high-pH-treated PM pellet raised the question of whether any hydrophobic tubulin could be recovered in the supernatant or if the hydrophobicity of the tubulin was lost. To answer this question, PMs without prior Brij 58 treatment were exposed to pH 12.0 and the subsequent pellet and supernatant were dialyzed to normal pH before Triton X-114 fractionation was conducted. Almost all of the tubulin was recovered in the supernatant (i.e. the tubulin was released by the high pH), and of this, most appeared in the aqueous

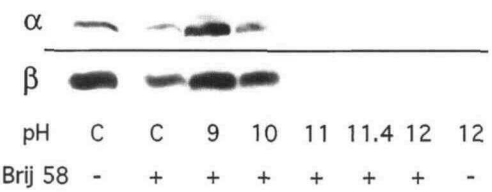

Figure 2. Immunoblot showing the presence of hydrophobic α- and β-tubulin in PM after extraction at different pH. Aliquots (approximately 0.2 mL) of inside-out PM, corresponding to 1 mg before Brij 58 treatment, were diluted to 6 mL with CO_3^- solutions at five different pH values and with control medium (C). After 30 min on ice, the PM vesicles were pelleted, resuspended in control medium to 1 mL, and fractionated with Triton X-114. For comparison, Triton X-114 fractionation was performed on 1 mg of pH 12.0-extracted, right-side-out PM (no prior Brij 58 treatment), and on 1 mg of untreated PM. Aliquots corresponding to approximately 40% of the detergent phases were run on SDS-PAGE followed by immunoblotting.

phase (Fig. 3). Some tubulin was recovered in the insoluble pellet but none in the detergent phase. The tubulin that remained in the PM pellet (7% α-tubulin and 14% β-tubulin) was recovered exclusively in the Triton X-114-insoluble pellet (Fig. 3). Thus, in this experiment the hydrophobic property of tubulin could not be recovered after the pH 12.0 wash, corresponding with the results of Beltramo and co-workers (1994).

High-Salt Extraction of the Triton X-114-Insoluble Pellet

The recovery of tubulin in the Triton X-114-insoluble pellets was always high, and some integral proteins (e.g. the major 28- to 30-kD polypeptide, tentatively identified as aquaporin; Johansson et al., 1996) were found only in these pellets and (surprisingly) not in the detergent phases (Fig. 4A). Upon repartitioning of the detergent-insoluble pellets with Triton X-114, no additional hydrophobic tubulin was released (not shown). This lack of release may have reflected the presence of detergent-resistant peripheral complexes, e.g. fractions of MTs and various MAPs associated with the PM. Such complexes may also include the hydrophobic tubulin, perhaps in association with integral proteins, which could explain why these were found in the detergent-insoluble pellet. If this detergent-insoluble material is held together to some extent by electrostatic interactions, as is the case between MTs and many MAPs, these interactions should be weakened at high ionic strength.

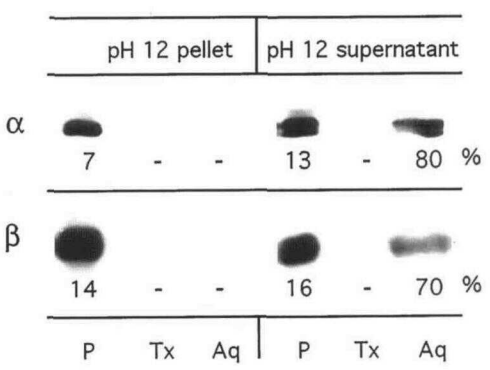

Figure 3. Immunoblot showing the distribution of α- and β-tubulin after Triton X-114 fractionation of pellet and supernatant after pH 12.0 extraction of PM. One milligram of right-side-out PM was washed at pH 12.0 as in Figure 2. The resulting pellet was resuspended in control medium to 1 mL, and both pellet and supernatant were dialyzed against control medium overnight. Triton X-114 was added to 0.5% (w/v) to both fractions and extraction was conducted as described in "Materials and Methods." Aliquots corresponding to 20(10)% (numbers in parentheses represent aliquots taken from fractions from the pH 12.0 supernatant) of the detergent-insoluble fraction (P), 45 (10)% of the detergent fraction (Tx), and 20 (1.5)% of the aqueous fraction (Aq) were subjected to SDS-PAGE followed by immunoblotting. The numbers in the figure represent the percentage of total α- or β-tubulin recovered, as determined by scanning software.

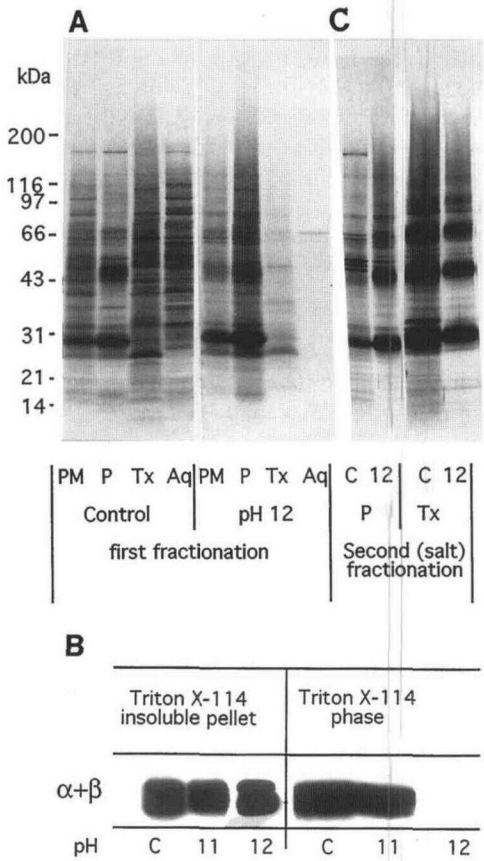

Figure 4. A, Silver-stained gel (5–20% acrylamide) of fractions obtained after Triton X-114 extraction of PM (control) and of PM (right-side-out) washed at pH 12.0 (fractions as in Figs. 1 and 3). B, Immunoblot showing the presence of tubulin in fractions obtained after reextracting Triton X-114-insoluble pellets in the presence of 0.7 M NaCl. The detergent-insoluble pellets obtained after Triton X-114 solubilization of inside-out (Brij 58-treated) PM, prewashed in control medium (C) at pH 11.0 and at pH 12.0 (as in Fig. 2), were resuspended in control medium including 0.7 M NaCl. Triton X-114 was added to 0.5% (w/v) and fractionation was repeated. Aliquots corresponding to 20% of the insoluble fractions and 40% of the detergent fractions were run on SDS-PAGE and then immunoblotted. Here, the blots were probed with combined α- and β-tubulin antibodies instead of using the antibodies separately on duplicate blots. C, Silver-stained gel (5–20% acrylamide) of control and pH 12.0 fractions as in B.

To test this hypothesis, the insoluble pellet from a Triton X-114 extraction of PM was repartitioned in the presence of 0.7 M NaCl. Also repartitioned were PM after a pH 11.0 and a pH 12.0 wash. A considerable amount of the α- and β-tubulin found in the Triton X-114-insoluble pellets from control as well as from pH 11.0-washed PM was released and recovered in the detergent phase (Fig. 4B). No hydrophobic tubulin could be released from the Triton X-114-insoluble pellet obtained from pH 12.0-washed PM (Fig. 4B). For all three treatments, tubulin still pelleted after this second Triton X-114 partitioning (Fig. 4B). In addition, the putative aquaporin at 28 to 30 kD (Johansson et al., 1996) was released from the Triton X-114-insoluble pellet to the detergent phase (Fig. 4C, control and pH 12.0).

Presence of Hydrophobic Tubulin in the Supernatant after pH 12.0 Wash

Although extraction at pH 12.0 had led to the absence of tubulin from the Triton X-114 phase in the experiments shown in Figures 2 and 4, these results did not necessarily mean that the high pH had broken an association between tubulin and a hydrophobic component (rendering the hydrophobic tubulin hydrophilic), as suggested by Beltramo et al. (1994). An alternative explanation could be that the hydrophobic component had been released together with tubulin upon pH 12.0 extraction but that the complex for some reason had not been recovered in the detergent phase in the subsequent Triton X-114 fractionations, and instead partitioned to the aqueous phase or pelleted with the detergent-insoluble material.

As seen in Figure 3, after pH 12.0 extraction tubulin was pelleted in the neutralized supernatant. This pellet was known to be the result of the neutralization rather than being induced by Triton X-114, since centrifugation after dialysis of the pH 12.0 supernatant but before inclusion of Triton X-114 pelleted similar amounts of tubulin (not shown). With PM pretreated with Brij 58 but not with untreated PM, hydrophobic tubulin could be recovered after extraction at pH 12.0 in the neutralized supernatant (Fig. 5), as if the presence of Brij 58 in the membrane during the pH 12.0 extraction prevented aggregation of hydrophobic tubulin into pelletable or hydrophilic complexes upon neutralization. The addition of Triton X-114 before neutralization instead of after did not change this result (Fig. 5).

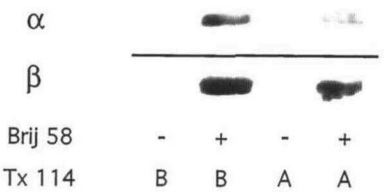

Figure 5. Immunoblot showing the presence of hydrophobic α- and β-tubulin in supernatants after pH 12.0 extraction of right-side-out and inside-out PM (with [+] or without [−] Brij 58), where Triton X-114 was added before (B) or after (A) neutralization. Isolated PM (2 mg) was diluted in control buffer with or without Brij 58 and pelleted. The pellets were resuspended in 6 mL of CO_3^- solution, pH 12.0, and incubated on ice for 30 min. After centrifugation, each supernatant was divided into two 3-mL samples. Triton X-114 was added before dialysis against control buffer (B) to one of each sample, and to the remaining two samples after dialysis (A). Triton X-114 fractionation was then performed as before, and samples corresponding to 15% of each detergent fraction were separated on gels and immunoblotted.

DISCUSSION

The cortical MTs of plant cells are important for cellulose microfibril deposition, thereby determining the expansion direction of a given plant organ. It was recently reported that intact MTs are also needed to allow regulation of PM-bound Ca^{2+} channels (Thion et al., 1996), which are involved in the regulation of Ca^{2+} homeostasis of plant cells. Also, the auxin carrier protein (*N*-1-naphthylphthalamic acid-sensitive) seems to be directly linked to the cytoskeleton (Cox and Muday, 1994). These are only some examples of the interactions between cytoskeletal components and the PM that are important for the regulation of cellular activities. The discovery that bridges between the PM and MTs can be seen under the microscope (Hardham and Gunning, 1978) implied that the MT link to PM is through specific proteins. These are probably under developmental regulation, so it is important to understand the biochemistry of such links and how this developmental regulation is achieved.

In this work we have characterized the interaction between tubulin and the PM in cauliflower using Triton X-114 fractionation. Based on marker-enzyme activities and scanned immunoblots, the percentage of total tubulin in the cauliflower homogenate (after a 10-min, 1000*g* centrifugation) that associated with the PM was approximately 15% (not shown). Although this figure is approximate, it shows that the PM-copurified tubulin represents a significant proportion of total cauliflower tubulin. One-half of the PM-copurified tubulin was found to be resistant to Triton X-114 and the other half was Triton soluble. When the Triton-soluble fractions were heated to 35°C, tubulin was recovered in both the detergent fraction and the aqueous fraction. The hydrophobic tubulin (enriched in the detergent fraction) seems to be characteristic of the membrane fractions because it was found in the PM and the ICM fraction but not in the cytosolic fraction. The presence of hydrophobic tubulin in the ICM fraction could be attributed to the PM content of this fraction. The specific activity of the PM marker enzyme, glucan synthase II, was roughly 10 times less in the ICM fraction than in the PM fraction (Sonesson and Widell, 1993).

Hydrophobic α- and β-tubulin constituted 12 and 16%, respectively, of the recovered PM-associated tubulin (Fig. 1). The actual percentage of PM-associated tubulin that is hydrophobic is probably at least double these amounts, because high-salt extraction of the detergent-insoluble pellet released a significant amount of tubulin to the detergent phase (Fig. 4B). It is possible that the detergent-insoluble pellet contained fragments of the PM-associated cytoskeleton and that hydrophobic tubulin was recovered here because of electrostatic interactions with such cytoskeletal fragments. This would help to explain why high salt was necessary to release hydrophobic tubulin from this pellet. Plant MAPs have been shown to interact with MTs in a

salt-sensitive manner (Nick et al., 1995; Chan et al., 1996; Marc et al., 1996), and it is possible that the weakening of such bonds resulted in the release of hydrophobic tubulin from the detergent-insoluble pellet. Sonobe and Takahashi (1994) showed that a salt-sensitive component was necessary for MT polymerization on protoplast ghosts from BY-2 cells.

High-salt treatment also weakened the association of the putative aquaporin at 28 to 30 kD (Johansson et al., 1996) to the insoluble pellet. The 28- to 30-kD integral protein was insoluble in Triton X-114 unless a high concentration of salt was added (Fig. 4, A and C).

The release of tubulin from the rat brain PM at high pH and the subsequent absence of tubulin from the detergent phase in the work of Beltramo et al. (1994) could be attributed to a dissociation of tubulin from a membrane component. In our work we present an alternative explanation to this absence of hydrophobic tubulin, the formation of Triton-insoluble aggregates upon neutralization of the pH 12.0 supernatant. The presence of Brij 58 in the PM fraction (Fig. 5) probably prevented the formation of such aggregates by shielding hydrophobic domains. Thus, with cauliflower PM at least, the hydrophobic property appears not to be lost as the tubulin is released from the membrane at high pH.

The hydrophobicity of a fraction of PM-associated tubulin could reflect a direct or indirect interaction of this tubulin with the lipid bilayer or with an integral membrane protein. Support for tubulin being associated (directly or through a link) with an integral membrane protein can be found in the association of MTs to PM in protoplast ghosts of BY-2 cells, where this association was inhibited if the protoplasts were pretreated with trypsin or chymotrypsin (Akashi and Shibaoka, 1991). It was suggested that the protease treatment affected the extracellular portion of a transmembrane protein, which in turn led to the loss of affinity of the intracellular domain for MTs.

Although high pH was effective at releasing hydrophobic tubulin from the PM, which points to a peripheral association, the hydrophobic behavior could also be explained by tubulin itself carrying a hydrophobic domain; it may be a covalently bound fatty acid or prenyl group. This would be consistent with the release of tubulin by high pH, since the hydrophobic component is quite small compared with the hydrophilic parts of the tubulin. Soluble proteins can become membrane associated by exposing lipid domains as part of a signal transduction mechanism. This lipid character can be enough to give the protein a hydrophobic behavior upon Triton X-114 fractionation, such as occurs with the acylated neuronal growth cone protein (Skene and Virág, 1989) and the prenylated small GTP-binding protein rab6b from mouse cells (Roa et al., 1993). Palmitoylation as a means of posttranslationally modifying tubulin was recently reported for platelets (Caron, 1997).

In conclusion, some of the tubulin from cauliflower PM displays hydrophobicity, even though it seems to be a peripheral protein. This hydrophobicity does not disappear when the tubulin is released from the membrane by high pH, indicating either that it is a property of the tubulin itself, or that there is a tight association between tubulin and an integral membrane protein. If the hydrophobicity of tubulin reflects a mode of anchoring of MTs to the PM, further elucidation of this property may explain many important cellular events.

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