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Ubiquitin is a component of the microtubule network

(immunofluorescence/microtubule-associated proteins/intracellular localization)

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ABSTRACT **Immunofluorescence microscopy was used to study the intracellular localization of ubiquitin. Baby hamster kidney cells (BHK cells) and several other cell lines were probed with a well characterized monoclonal antibody to ubiquitin. The antibody stained a complex cellular structure that we identified as the microtubule network. The anti-ubiquitin antibody bound to the microtubule network at all stages of the cell cycle, and we showed that the apparent association of ubiquitin with the microtubule network is not an artifact of crosslinking of free ubiquitin to the cell structure. Immunoblot procedures demonstrated that tubulin itself was not ubiquitinated. We propose that ubiquitin and/or ubiquitin-protein conjugates are associated with those networks as a new class of microtubule-associated protein. The targeting of ubiquitin to specific sites within the cell by its association with the microtubule network may regulate some of the functions of ubiquitin.**

Ubiquitin (Ub) is a protein of 76 amino acid residues that is present in every eukaryotic cell (1, 2). The sequence of this molecule is invariant in animal cells from insects to humans (2), and it is likely that Ub has been conserved because it serves essential functions common to all eukaryotic cells. Ubiquitin is found in cells both as a free molecule and conjugated to other proteins via a peptide or isopeptide bond between its COOH-terminal residue and α- or ϵ-NH_2 groups of the target proteins (2, 3), and it is likely that Ub serves an important function as part of the Ub-protein conjugates. Whether the free form of Ub is only an intermediate on the pathway to its conjugated state or plays a direct role in other cellular events as an unconjugated species is unknown.

There appear to be two general classes of ubiquitinated proteins. In one class are those conjugates that are rapidly degraded via a nonlysosomal ATP-dependent pathway (4, 5). How Ub functions to target proteins for rapid degradation is not understood. In the other class are specific ubiquitinated proteins that are not rapidly degraded. These include the nuclear proteins, histones H2A and H2B (3, 6), and some plasma membrane proteins, including the lymphocyte homing receptor (7, 8) and the receptor for the platelet-derived growth factor (9). Ubiquitination of these proteins is selective; other histones and other well-characterized membrane proteins are not ubiquitinated (V.A.F. and H.T.S., unpublished data). That one class of ubiquitinated proteins is rapidly degraded whereas another class is not suggests that Ub serves more than one function. It is also possible that the localization of these conjugates influences the roles Ub plays.

Recently we reported that Ub has intrinsic proteolytic activity and proposed that the conjugation of Ub to a protein can convert that conjugate into an *ad hoc* protease (10). Thus, one function of Ub in a conjugate might be proteolytic processing in *cis*, which could initiate degradation of the conjugate, or in *trans*, which could proteolytically process a component interacting with the conjugate. In either case, we anticipate that other cellular factors are involved in regulating the proteolytic activity of Ub.

As noted, Ub-protein conjugates that are rapidly degraded appear to be cytosolic, whereas the stable conjugates are compartmentalized. This raises the possibility that cellular localization plays a role in regulating the function of the Ub-protein conjugate and may regulate its proteolytic activity as well. To study the subcellular distribution of Ub and Ub-protein conjugates, we probed several cultured cell lines by indirect immunofluorescence using a well-characterized monoclonal antibody to Ub (7, 8, 11). This mAb, 1-2H11, binds to purified Ub in a solid-phase microtiter assay and on immunoblots but does not recognize soluble free Ub with a high enough binding constant for direct immunoprecipitation analysis. It also recognizes specific Ub-protein conjugates on immunoblots and by immunoprecipitation (8, 11). The epitope recognized by 1-2H11 is contained in the amino acid sequence of Ub, residues 34–54 (7, 11). This sequence is apparently unique to Ub; thus, the antibody is a highly selective immunochemical probe for Ub. Our immunofluorescence studies using this monoclonal antibody (mAb) suggest that Ub is a previously unrecognized component of the microtubule network.

MATERIALS AND METHODS

Ub and mAb to Ub. Ub was either purified from outdated human erythrocytes (10) or obtained already purified from bovine erythrocytes (Sigma). The Ub in these preparations was found to be identical by sequence analysis, high resolution peptide mapping, and isoelectric focusing (data not shown). Both preparations were composed of >95% complete chain, residues 1–76, with the remaining fraction (<5%) missing only the COOH-terminal glycylglycine; thus, these preparations were used interchangeably.

The mouse hybridoma, 1-2H11, producing mAb to Ub, was prepared by standard fusion and screening protocols and has been used in earlier studies (7, 8, 11). The complete details of its characterization will be reported elsewhere. Hybridomas were grown in culture, and culture supernatants were used as the source of antibody in all experiments.

A solid-phase radioimmunoassay was used to titer the antibody against purified Ub. The assay was carried out by sequentially drying Ub on a microtiter plate (Falcon, 3911), blocking in B buffer [20 mM Tris·HCl/154 mM NaCl/0.02% Na azide/2% (wt/vol) casein (Sigma)], incubating with mAb 1-2H11 diluted in B buffer, incubating with rabbit anti-mouse IgG (affinity-purified, heavy and light chain-specific) (Cappel Laboratories, Cochranville, PA), and incubating with ^{125}I-labeled protein A (ICN; ^{125}I-protein A) diluted in B buffer. Individual wells were cut out with scissors, and specifically bound ^{125}I-protein A was detected by γ counting (Nuclear-

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Abbreviations: Ub, ubiquitin; mAb, monoclonal antibody; BHK cells, baby hamster kidney cells.

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Chicago). Between each step the wells were washed extensively with B buffer.

Preadsorption of 1-2H11 culture supernatants was performed in microtiter plates coated with an excess of pure Ub. After incubating the culture supernatants in the antigen-coated wells for 18 hr, the supernatant fluids were recovered. These preadsorbed supernatants had titers 1/500th that of the unadsorbed supernatants. Adsorption of the culture supernatants in microtiter plates blocked with B buffer alone did not affect titers.

Immunofluorescence Analyses and Electron Microscopy. Baby hamster kidney cells (BHK cells) were grown on 20-mm^2 glass coverslips in Eagle's medium supplemented with 10% fetal calf serum.

Method 1. Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline at 21°C for 20 min, and the membranes were made permeable by immersing in acetone for 5 min at −20°C. The fixed, permeabilized cells were washed with phosphate-buffered saline and incubated with the primary antibody (hybridoma culture supernatants diluted in phosphate-buffered saline) for 45 min at 31°C, rinsed with buffer, and then incubated with fluorescein-conjugated goat anti-mouse Ig (Miles Laboratories) for 45 min. Cells were washed with phosphate-buffered saline and water and then air-dried. Coverslips were mounted on glass slides with *p*-phenylenediamine in glycerol (12), and the slides were viewed in a Zeiss-IM35 microscope equipped with epifluorescence optics. Photographs were taken on Kodak Tri-X pan film.

Method 2. The BHK cells were first extracted with Triton X-100 to prepare the cytoskeletons before fixation (13). The cells on coverslips were washed in PHEM buffer (60 mM Pipes/25 mM Hepes/10 mM EGTA/2 mM $MgCl_2$, pH 6.8), extracted for 1 min with 0.15% Triton X-100 in PHEM buffer, washed, and then fixed for 20 min with 3.7% formaldehyde in PHEM buffer. All operations were at 21°C. The extracted and fixed cells were incubated with the primary antibody and then with the fluorescein-labeled second antibody as above (method 1) for immunofluorescence studies. For electron microscopy, the cells were grown on gold grids (14), extracted with Triton X-100, fixed with formaldehyde, and treated with the primary antibody as described above. The extracted cells were next incubated with second antibody conjugated with 5-nm gold particles (15) and viewed in the electron microscope.

Immunoblotting. BHK cells were grown in minimal essential medium with Hanks' salts containing 10% fetal calf serum, penicillin (100,000 units/liter), streptomycin (100 mg/liter), and kanamycin (50 mg/liter) at 37°C in 5% CO_2/95% air. Cells were harvested from T150 flasks (Costar, Cambridge, MA) during logarithmic-phase growth by treating with 0.25% trypsin and 0.05% EDTA in Hanks' balanced salt solution. Detached cells were pelleted by low-speed centrifugation and washed four times with phosphate-buffered saline at 4°C. Whole-cell lysates were prepared by adding 5 volumes of 7 mM sodium phosphate (pH 7.4) containing 0.25 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 0.25 mM *N*-α-tosyllysine chloromethyl ketone, 0.25 mM phenylmethylsulfonyl fluoride, 5 mM *N*-ethylmaleimide, and 5 mM EDTA to the cell pellet. To this mixture were added 5 volumes of sample buffer [14.3 mM Tris·HCl, pH 7.5/14.3 mM EDTA/14.3% (vol/vol) glycerol/10.7% (wt/vol) lithium dodecyl sulfate ($LiDodSO_4$)/14.3 mM dithiothreitol]. The entire mixture was sonicated to disrupt the chromatin and boiled for 3 min. Purified Ub was mixed in equal volume of sample buffer. Proteins were fractionated by $NaDodSO_4$/PAGE on 10–20% linear gradient polyacrylamide gel (16). Protein molecular mass standards were run on adjacent lanes. Proteins in gels were either stained with Coomassie brilliant blue R-250 or transferred to Immobilon PVDF membrane (Millipore) as described (17). After transfer, the membranes were incubated in B buffer for 1 hr at 37°C, and sections of membrane were incubated overnight at ambient temperature with the primary mouse mAbs (diluted in B buffer). These membranes were washed with B buffer, incubated with affinity-purified rabbit anti-mouse IgG (heavy and light chain-specific; Cappel Laboratories), washed with B buffer, and finally incubated with ^{125}I-protein A (1×10^6 dpm/ml in B buffer) for 2 hr at room temperature to identify immunocomplexes. The washed membranes were dried and autoradiographed (Kodak X-Omat AR film).

RESULTS

mAb Specific to Ub Binds to the Cytoskeleton. We used a well-characterized mAb to Ub, 1-2H11, to study the intracellular localization of Ub in cultured BHK cells. When the cells were fixed with formaldehyde, permeabilized with acetone, and incubated with the primary antibody 1-2H11 followed by incubation with the fluorescein-labeled second antibody, intense fluorescence was observed on a well-defined intracellular network that radiated from the perinuclear region into the cytoplasm (Fig. 1*B*). This staining was so intense that the nuclear staining appeared to be relatively weak. The staining pattern with the mAb to Ub was not due to nonspecific binding of the first (mAb to Ub) or second (fluorescein-conjugated polyclonal antibody to mouse Ig) antibody because this network was not seen when cells were treated with: (*i*) the 1-2H11 first antibody preadsorbed with Ub (Fig. 1*A*), (*ii*) the control first antibody (mAb to a non-Ub protein) (Fig. 1*C*), or (*iii*) the second (fluorescein-conjugated) antibody alone (data not shown). Finally, 1-2H11 showed the same staining pattern in 3T3 and fat head minnow cells (data not shown).

Ub Is Associated with the Microtubule Network. The cytoskeletal network stained by mAb to Ub resembles the microtubule network (18). To verify that 1-2H11 was in fact decorating microtubules, we used two approaches. First, we disrupted the microtubule network of BHK cells with colchicine and then stained the cells with either anti-tubulin or 1-2H11. Colchicine abolished the typical staining pattern of anti-tubulin (Fig. 2*B*) as well as that of 1-2H11 (Fig. 2*A*). Second, we performed a double immunofluorescence analysis to determine the staining pattern with the anti-Ub mAb and anti-tubulin antibody in the same cell. The staining patterns of 1-2H11 (Fig. 2*C*) and anti-tubulin (Fig. 2*D*)

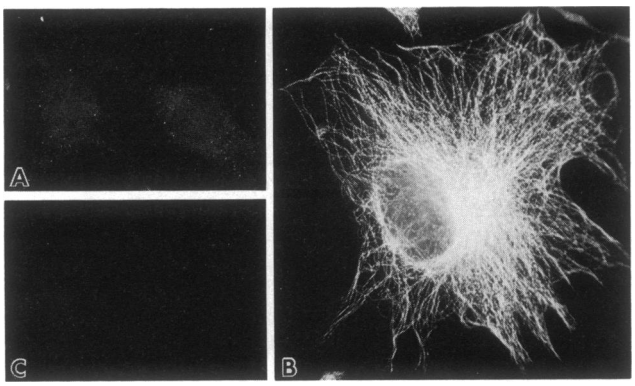

FIG. 1. mAb to Ub, 1-2H11, binds to a cytoskeletal structure. Immunofluorescence micrographs of BHK cells that were fixed and made permeable by method 1. (*A*) 1-2H11 culture supernatants were adsorbed with purified Ub before incubation as first antibody. (*B*) 1-2H11 culture supernatant as first antibody. (*C*) 5-1A6 (mAb to the human erythrocyte anion transport protein) culture supernatant as first antibody.

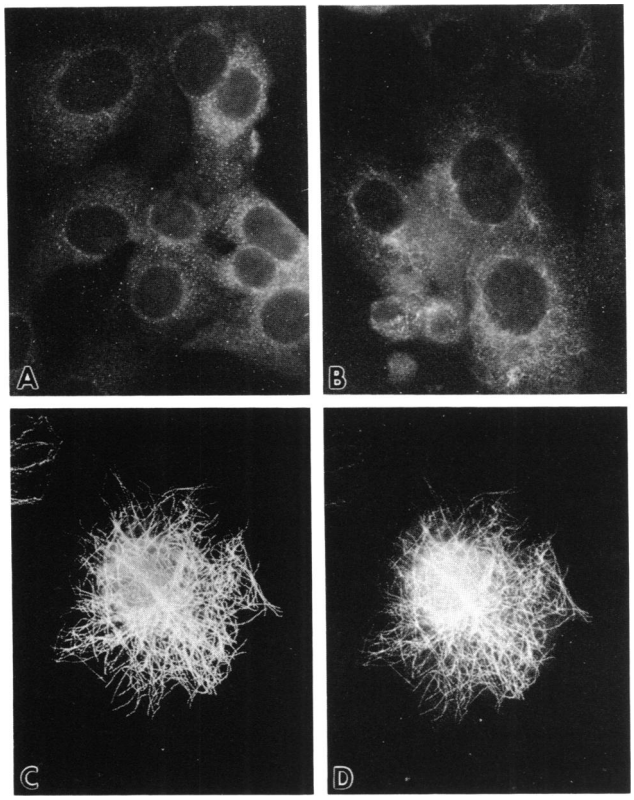

FIG. 2. mAb to Ub binds to the microtubule network. (*A* and *B*) Immunofluorescence micrographs of BHK cells treated with 10 μg of colchicine per ml for 2 hr and then fixed and permeabilized by method 1. In *A*, 1-2H11 was the first antibody; in *B*, rabbit anti-tubulin antibody was used (Polyscience, Warrington, PA). (*C* and *D*) Immunofluorescence micrographs derived from a permeabilized BHK cell double-stained with 1-2H11 and anti-tubulin antibody. For double staining, the cells were incubated with 1-2H11 and then fluorescein-conjugated goat anti-mouse antibody. The cells were washed with phosphate-buffered saline and incubated with rabbit anti-tubulin antibody and rhodamine-conjugated goat anti-rabbit antibody. Cells were viewed by using the fluorescein filter to observe 1-2H11 staining (*C*) and the rhodamine filter to observe the anti-tubulin staining (*D*).

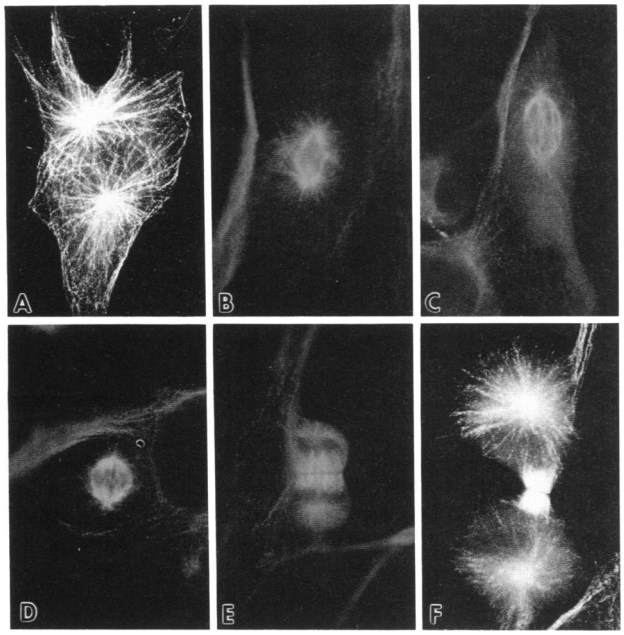

FIG. 3. mAb to Ub stains the microtubule network at all mitotic stages. Immunofluorescence micrographs are of BHK cells that were fixed and permeabilized and then stained with the anti-Ub mAb, 1-2H11. Fields of cells were examined under the fluorescence microscope, and examples of the different known mitotic forms were selected to prepare the collage shown.

coincided. These experiments confirmed that the mAb to Ub, 1-2H11, binds to the microtubule network.

To determine whether 1-2H11 specifically stained only the cytoplasmic microtubule network or if it also stained other microtubule-containing structures (e.g., asters and mitotic spindles), we examined the staining pattern of mitotic cells (Fig. 3). In different stages of prophase (Fig. 3 *A*, *B*, and *C*), the antibody brightly stained the asters (Fig. 3*A*) and the mitotic spindle (Fig. 3 *B* and *C*). In metaphase cells, the spindle showed fluorescence but the chromosomes did not; the chromosomes appeared as dark unstained regions at the equatorial plate (Fig. 3*D*). Cells in telophase showed highly fluorescent microtubules on either side of the cytoplasmic constriction ("interzonal fibers") (19), nonfluorescent chromosomes near the poles, and fluorescent spindle poles (Fig. 3*E*). Fig. 3*F* shows two daughter cells connected by an intensely fluorescent cytoplasmic bridge of microtubules and brightly fluorescent asters at the poles. Thus, in all mitotic stages examined, the fluorescence due to 1-2H11 binding is similar to that observed with anti-tubulin (18, 19). These results show that 1-2H11 recognizes all microtubule-containing structures and suggest that Ub is associated with microtubules throughout the cell cycle. A decrease in total fluorescence due to 1-2H11 binding was seen in the cells as they progressed through the mitotic cycle, but we were unable to determine if the decreased fluorescence was due to qualitative or quantitative changes in the association of Ub with the microtubules or due to changes in the organization of the microtubules.

The Association of Ub with the Microtubule Network Is not an Artifact of Fixation. Ubiquitin is present in both free and conjugated forms in the cytosol and nuclear fractions (20). In our first studies, fixation of the cells preceded membrane permeabilization, and thus the apparent association of Ub with the microtubule network could have resulted from crosslinking of free Ub to the cellular structures. This possibility was tested by first extracting the cells with Triton X-100 under conditions wherein the microtubule network remained intact while the bulk of the cytosolic components were extracted (13); the remaining cellular structures were washed and fixed for immunostaining. Preextraction of the cells with Triton X-100 before fixation preserved the microtubule network, as visualized by binding of anti-tubulin antibody (Fig. 4*B*), and also preserved the structure decorated by the anti-Ub antibody (Fig. 4*A*). The association of 1-2H11 with the microtubules was further confirmed by immunogold labeling at the electron microscopic level. The Triton-extracted cells were treated with anti-Ub mAb, 1-2H11 (Fig. 4*C*), or anti-tubulin antibody (Fig. 4*D*) and then incubated with second antibody conjugated with gold particles. In the electron microscope, the gold particles were seen over the microtubules but not on the intermediate filaments or the microfilaments. These results strongly support the notion that Ub is a component of the microtubule network and is not present as an artifact of fixation.

Tubulin Is not a Ubiquitinated Protein. The similar immunostaining by anti-tubulin antibody and anti-Ub antibody could be explained if a fraction of the tubulin were itself ubiquitinated. As one test of this hypothesis, we analyzed BHK cells by $NaDodSO_4$/PAGE and immunoblotting procedures (Fig. 5). Monoclonal antibody 1-2H11 recognized purified Ub (Fig. 5, lane 6) and free Ub in the total BHK cell lysate (lane 5). In addition, 1-2H11 recognized Ub-protein conjugates of 20, 22, 23, 41, 47, 53, 87, and 94 kDa. Longer exposure of the autoradiogram showed that many more

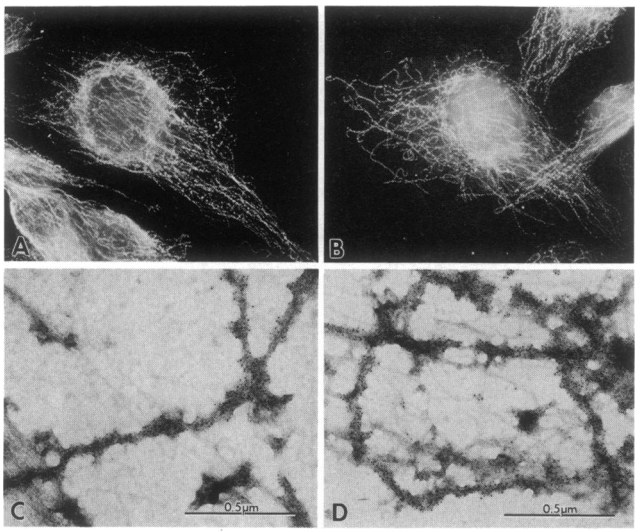

FIG. 4. The binding of anti-Ub antibody to the cytoskeletons is not an artifact of fixation. BHK cells were extracted with Triton before fixation by method 2. (*A* and *C*) 1-2H11 as first antibody. (*B* and *D*) Anti-tubulin as first antibody. The immunofluorescence micrographs (*A* and *B*) were prepared by binding the first antibody with a fluorescein-labeled second antibody. The electromicrographs (*C* and *D*) used a gold-labeled second antibody as described.

Ub-protein conjugates were present in these cells. These ubiquitinated proteins do not correspond to major protein species detected by Coomassie blue staining of a portion of the gel before electroblot (Fig. 5, lane 1); thus, they are apparently less abundant proteins. No bands were observed when the second antibody and ^{125}I-protein A were used alone (Fig. 5, lanes 3 and 4) or when control hybridoma culture supernatants were used as first antibody (data not shown).

The major Ub-protein-conjugated species, the 22-kDa band, is the ubiquitinated histone H2A (3) because it comigrated with the ubiquitinated histone purified from calf thymus (HTS and VAF, data not shown). The identities of the other major Ub-protein conjugates are not known at this time.

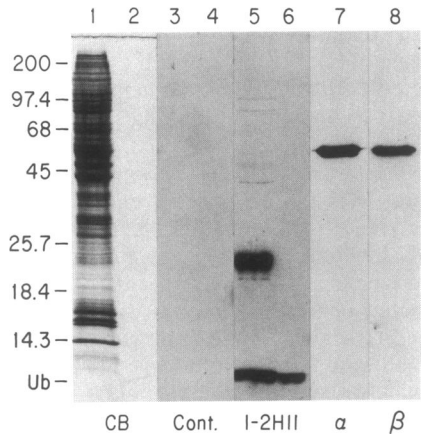

FIG. 5. α- and β-tubulin are not ubiquitin conjugates. BHK cells were solubilized in LiDodSO$_4$ and fractionated by NaDodSO$_4$/PAGE on a 10–20% linear gradient acrylamide gel (lanes, 1, 3, 5, 7, and 8). Each lane contained an extract from 3×10^5 cells. Purified Ub was run on adjacent lanes (lanes 2, 250 ng; lanes 4 and 6, 50 ng). Lanes 1 and 2 were stained with Coomassie blue (CB), and the remaining gel was electroblotted to Immobilon PVDF membranes. The immunoblots were incubated with a rabbit anti-mouse antibody and ^{125}I-protein A. The autoradiograms are shown in lanes 3–8: 3 and 4, incubation with second antibody alone [control (Cont.)]; 5 and 6, incubation with anti-Ub mAb 1-2H11; 7, incubation with a mouse monoclonal antibody to α-tubulin (N356, Amersham); and 8, incubation with anti-β-tubulin (N317, Amersham).

Both α- and β-tubulin appear as 55-kDa proteins in this gel system (12) and electroblots of total LiDodSO$_4$-solubilized BHK cells probed with anti-α-tubulin (Fig. 5, lane 7) or anti-β-tubulin (Fig. 5, lane 8) showed only a single protein species of 55 kDa. If tubulin were ubiquitinated, higher molecular mass forms would have been observed, and none were detected even in overexposed autoradiograms of the immunoblots. Conversely, 1-2H11 did not react with a 55-kDa protein (Fig. 5, lane 5), demonstrating that tubulin does not directly cross-react with 1-2H11. These results strongly suggest that the immunoreactivity of the microtubule network with 1-2H11 is not due to Ub covalently conjugated to tubulin but to the specific binding of free Ub or ubiquitinated microtubule-associated proteins (MAPs) to the tubulin network.

DISCUSSION

Because Ub has been found conjugated to specific histones and cell-surface molecules, it is clear that it is present in different cellular compartments. The general cellular distribution was studied by microinjection of ^{125}I-labeled Ub into cells followed by subcellular fractionation (20), and indeed, both free and conjugated Ub were present in the cytosol and nuclear fractions. Our results using immunofluorescence analysis and immunoelectron microscopy demonstrate for the first time that a fraction of the Ub, by association with the microtubule network, can be localized to specific sites within the cell.

Because mAb 1-2H11 specifically recognizes Ub, the binding of 1-2H11 to the microtubule network implies that Ub is a component of this structure. Ub may bind noncovalently to assembled microtubules. Alternatively, a subset of the microtubule-associated proteins may be Ub conjugates. However, it is possible that there is another, as yet uncharacterized, protein that cross-reacts with this antibody.

The identity of the Ub-species bound to the microtubules may provide insight into the function of this association. Although the intense staining of the microtubules with 1-2H11 suggests that the form(s) of Ub bound to the microtubules is an abundant species, this form may not appear as a major band on immunoblot analysis for several reasons. Electrophoretic immunoblotting is not quantitative, and all Ub-conjugates may not transfer or be captured on the membrane with equal efficiency. Furthermore, the presentation of the Ub-epitope recognized by 1-2H11 is affected by both the specific conjugate and the membrane matrix used in blotting (7, 11). Whether the association of Ub with the microtubules is due to the binding of one of the Ub-conjugates observed in the immunoblot (Fig. 5), of a conjugate not detected in these experiments, or of free Ub will be determined as these components are purified and reconstituted *in vitro*.

While qualitative information on localization can be obtained from immunofluorescence microscopy, the actual distribution of components within the cell cannot be determined by this technique. Thus, it is unclear whether the relatively weak 1-2H11 staining of the nucleus is due to diffuse distribution of the ubiquitinated histones, which are strongly recognized on immunoblots (Fig. 5), or due to some interference with *in situ* antibody binding. For example, the epitope of Ub recognized by 1-2H11 could be masked when the ubiquitinated histones are in the chromatin. On the other hand, the intense staining of the microtubules demonstrates only that these binding sites are clustered. It is possible that other cellular structures are highly ubiquitinated but are not visible under these conditions.

1-2H11 is not the only antibody to Ub that binds to the microtubule network; we have found that 3 of 9 polyclonal antibodies to Ub and 4 of 10 mAbs to Ub also decorate the cytoskeleton, with several staining the nucleus as well (not shown). However staining with these antibodies also produced diffuse background fluorescence that obscures, to various degrees, the fine structure of the cytoskeletal staining. We believe that these antibodies may bind *in situ* to a wider population of ubiquitinated cellular components than does 1-2H11. This selective recognition of Ub-conjugates by different mAbs has been noted (7, 8, 10, 11).

It has been reported that Ub is associated with structures that appear in the degenerating neurons that are characteristic of Alzheimer's disease (21, 22). These structures, neurofibrillary tangles, are composed in part of paired helical filaments and contain several components including some microtubule-associated proteins that are known to be associated with normal brain microtubules (23). However, the biochemical composition of these structures is ill-defined, and the reason they accumulate in diseases associated with mental dysfunction is not known. The association of Ub with these structures may be important to the disease process. For example, the specific ubiquitination of a new component or aberrant ubiquitination of a normal structural component may be involved in the development of these disorders. Alternatively, ubiquitin's presence in these pathological structures may be irrelevant to the cause of the disease but result from a reorganization of preexisting Ub-protein conjugates that are normally associated with the cytoskeleton. Our observation that Ub is associated with the microtubule network in normal cells suggests that this second explanation may be correct.

Although our study shows that some fraction of the Ub is localized in cells by association with the microtubules, the physiologic significance of this observation is unclear. Since we recently reported that Ub has intrinsic proteolytic activity (10), the association of Ub with the microtubules suggests that proteolytic processing might be a cytoskeletal function. Whether this processing is involved in known microtubule functions (e.g., chromosome segregation, cytokinesis) or implies novel functions for the microtubules in cellular events remains to be explored.

After this manuscript was submitted, Ball *et al.* (24) reported that a stable form of ubiquitinated actin is present in *Drosophila* flight muscle. Although this Ub conjugate could be unique to this highly specialized cell type, it suggests that Ub is not only a component of the microtubule network but may play a broader role in cytoskeletal function.

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