Immunolocalization and Molecular Properties of a High Molecular Weight Microtubule-bundling Protein (Syncolin) from Chicken Erythrocytes

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Abstract. A protein of apparent molecular weight 280,000 (syncolin), which is immunoreactive with antibodies to hog brain microtubule-associated protein (MAP) 2, was purified from chicken erythrocytes. Immunofluorescence microscopy of bone marrow cells revealed the presence of syncolin in cells at all stages of erythrocyte differentiation. In early erythroblasts syncolin was diffusely distributed throughout the cytoplasm. At later stages it was found along microtubules of the marginal band, as confirmed by immunoelectron microscopy. The association of syncolin with the marginal band was dependent on the integrity of microtubules, as demonstrated by temperature-dependent de- and repolymerization or marginal band microtubules. Syncolin cosedimented in a saturable manner with microtubules assembled in vitro, and it was displaced from the polymer by salt. Brain as well as erythrocyte microtubules, reconstituted with taxol from MAP-free tubulin and purified syncolin, were aggregated into dense bundles containing up to 15 microtubules, as determined by electron microscopy. On the ultrastructural level, syncolin molecules were visualized as globular or ringlike structures, in contrast to the thin, threadlike appearance of filamentous MAPs, such as brain MAP 2. According to ultrastructural measurements and gel permeation chromatography, syncolin's molecular weight was $\sim 1 \times 10^6$. It is suggested that syncolin's specific function is the crosslinking of microtubules in the marginal band and, by implication, the stabilization of this structure typical for nucleated (chicken) erythrocytes.

MICROTUBULES, one of three prominent filamentous elements of the cytoskeleton, consist of tubulin, their main subunit component, and a number of associated proteins, called microtubule-associated proteins (MAPs).[1] In brain, where microtubules are most abundant, two major classes of MAPs have been identified: fibrous MAPs, comprising high molecular weight MAPs (apparent $M_r > 280,000$), 200-kD MAPs, and tau proteins (apparent $M_r = 55,000–68,000$), and energy-transducing MAPs, including kinesin and cytoplasmic dynein (for a recent review see Wiche et al., 1991). Energy-transducing MAPs are considered to mediate various microtubule-dependent motile phenomena, while the proposed major functions of fibrous MAPs involve microtubule crosslinking and stabilization. Contrary to energy-transducing MAPs, which are under study using a variety of different tissues and cell types, relatively little is known about the structure and function of fibrous MAPs from sources other than brain. Such studies would seem important, however, considering that structural diversities of MAP species are probably responsible for differential functions of these proteins, in particular their ability to bind to various tubulin isoforms or other cellular interaction partners.

Nucleated erythrocytes are an attractive source for non-neuronal microtubule proteins. Subjacent to the plasma membrane of these cells there is a circumferential ring of compactly bundled microtubules, called the marginal band, which is responsible, at least in part, for the generation of an asymmetric cell shape (Barrett and Dawson, 1974). Lately the biochemistry and the ultrastructure of marginal band microtubules have been studied extensively. Cohen and co-workers (1982) isolated intact marginal bands from dogfish erythrocytes by detergent and elastase treatment and found four tubulin-like proteins, but no MAPs. On the other hand, an anti–MAP 2-immunoreactive, high molecular weight protein, which was suggested to play a role in microtubule crosslinking, was identified in the marginal band of amphibian and avian erythrocytes (Sloboda and Dickersin, 1980; Centonze et al., 1985; Centonze and Sloboda, 1986). Murphy and co-workers identified a β-tubulin variant from chicken, specifically contained in erythrocytes and thrombocytes, that exhibited 17% overall divergence in its amino acid sequence compared with other chicken β-tubulins (Murphy and Wallis, 1983a; Murphy et al., 1987). Furthermore, the association of a 67-kD tau protein with the marginal band of chicken erythrocytes and the immunofluorescent staining of the marginal band using a rabbit antibody to hog brain MAP 2 were recently demonstrated (Murphy and Wallis, 1983b, 1985). However, only trace amounts of

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1. *Abbreviation used in this paper:* MAP, microtubule-associated protein.

MAP 2–related proteins were found in extracts of chicken erythrocytes by immunoblotting, and no MAP 2 was observed in preparations of in vitro assembled microtubules fractionated by SDS-PAGE (Murphy and Wallis, 1983b).

Here we report the isolation and partial characterization of a chicken erythrocyte high molecular weight (280,000) MAP that is associated with marginal band microtubules. Because of its specific microtubule bundling activity, as demonstrated in vitro, it is suggested that this protein be called syncolin, in reference to the Greek word for sticking together.

Materials and Methods

Preparation of Microtubule Proteins from Brain Tissues

Microtubule proteins were prepared from hog or chicken brain by two cycles of temperature-dependent polymerization and depolymerization according to the method of Karr et al. (1979). Purified tubulin and total MAP fractions were prepared by chromatography of microtubule proteins on phosphocellulose columns (Whatman, Maidstone, UK) (Weingarten et al., 1975). Hog brain MAP 2 was separated from MAP 1 by chromatography on hydroxyapatite (Calbiochem Corp., La Jolla, CA) (Kuznetsov et al., 1981) and further purified by DEAE-Sepharose CL-6B chromatography (Pharmacia, Uppsala, Sweden) (Foisner and Wiche, 1985). Purified proteins were used fresh for experiments or frozen in liquid nitrogen and kept at −70°C until use.

Purification of Tubulin and Syncolin from Chicken Erythrocytes

Blood from 4–5-wk-old chickens was obtained by decapitation of animals and collection of blood in beakers containing sodium citrate, pH 7.4, and sodium chloride at final concentrations of 1 and 0.9%, respectively. Red cells were collected by centrifugation at 1,000 g for 10 min at room temperature. The supernatant and the buffy coat, consisting mainly of leukocytes, were removed by aspiration. After washing in 0.9% sodium chloride the red cells were centrifuged and the pellets were resuspended in an equal volume of ice-cold 100 mM MES, pH 7, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.52 M sucrose, 1 mM PMSF, 5 mM DTT, and 1 mM GTP (buffer A). The cells were then disrupted by sonication (five times for 30 s, at 60-s intervals) using a microtip sonicator (Branson Sonifier, Danbury, CT) at 6/7 maximum output. Soluble cell extracts were obtained from these homogenates by centrifugation at 70,000 g for 45 min at 4°C. For polymerizing microtubules by temperature-dependent assembly, the protocol of Karr et al. (1979) was followed. Purified tubulin was obtained by chromatography of twice-cycled microtubule proteins on phosphocellulose columns (Weingarten et al., 1975). For taxol-driven assembly of microtubules, the soluble cell extracts were supplemented with GTP and taxol to final concentrations of 1 mM and 10 μM, respectively, and incubated for 30–45 min at 37°C. Polymerized microtubules were collected by centrifugation at 70,000 g for 60 min at 37°C.

To extract syncolin, taxol-polymerized microtubules were suspended in one-eighth of the extract volume of ice-cold 100 mM Pipes, pH 6.6, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM GTP, and 10 μM taxol (buffer B), homogenized in a glass homogenizer, and kept on ice for 30 min. The suspension was then centrifuged at 40,000 g for 60 min at 4°C. The resultant supernatant (4 ml) was overlaid onto a continuous gradient of 5–25% sucrose in 35 ml of 100 mM MES, pH 6.7, 1 mM EGTA, and 1 mM $MgCl_2$ (buffer C) and centrifuged at 95,000 g for 14 h at 4°C in an SW 28 rotor (Beckman Instruments, Inc., Palo Alto, CA). 1.5-ml fractions were collected by aspiration of the gradient from the bottom of the centrifuge tubes and fractions were analyzed on SDS 7.5% polyacrylamide gels (Laemmli, 1970). Fractions containing syncolin were pooled and subjected to chromatography on a hydroxyapatite column (bed volume: 0.5 × 5 cm) equilibrated with 50 mM Na_2HPO_4, pH 6.7, 0.5 mM $MgCl_2$ and 0.1 mM PMSF (buffer D). The column was washed with 0.5 M NaCl in buffer D, followed by buffer D. Step elution of proteins was done with 160 and 300 mM Na_2HPO_4, pH 6.7, both containing 0.5 M $MgCl_2$, and 0.1 mM PMSF. 0.5-ml fractions were collected and analyzed on SDS 7.5% polyacrylamide gels. Fractions containing syncolin were applied to a DEAE-Sepharose CL-6B column

(bed volume: 0.5 × 5 cm) equilibrated with buffer C plus 0.1 mM PMSF. After the column was washed with buffer C, adsorbed proteins were eluted with buffer C containing 0.1 mM PMSF and 0.5 M NaCl. For all experiments, preparations of syncolin were generally dialyzed against 25 mM MES, pH 6.7, 1 mM EGTA, 1 mM $MgCl_2$, and 0.1 mM PMSF at 4°C for 3 h.

Immunoreagents

Antibodies to hog brain MAP 2 or to syncolin from chicken erythrocytes were raised in rabbits using crushed gel pieces containing the immunogens after their purification by preparative SDS-PAGE. The specificity and titer of the antisera were routinely assessed by immunoblotting; there was no crossreactivity of the anti–MAP 2 antiserum with any of the MAP 1 subcomponents from hog brain. Affinity purification of anti-MAP 2 antibodies by adsorption to purified hog brain MAP 2 coupled to Affi-Gel 15 (Bio-Rad Laboratories, Inc., Richmond, CA) (method a) was done according to the instructions provided by the manufacturer. Bound antibodies were eluted with 200 mM glycine/HCl, pH 2.6, and 150 mM NaCl, followed by immediate neutralization with 1 M Tris. Affinity purification of anti-syncolin or anti-MAP 2 antibodies by adsorption to and elution from electrophoretically purified syncolin and hog brain MAP 2, respectively, immobilized on nitrocellulose sheets (method b) was performed following published procedures (Olmsted, 1981; Wiche et al., 1984).

Electrophoresis and Immunoblotting

Proteins were electrophoretically separated on SDS 7.5% polyacrylamide gels (Laemmli, 1970). Electrotransfer to nitrocellulose sheets (15 h at 300 mA, followed by 3 h at 800 mA) was done in 25 mM Tris, pH 8.3, 192 mM glycine, and 10% methanol (Towbin et al., 1979) at room temperature. The nitrocellulose sheets were blocked with 1% milk powder in 10 mM Tris/HCl, pH 8, and 150 mM NaCl (TBS) for 2 h, before 60-min incubations with various antibodies dissolved in TBS plus 0.2% Tween 20. Bound antibodies were detected using secondary antibodies conjugated to alkaline phosphatase and color development (Proto-Blot Immunoscreening System) according to the instructions provided by the manufacturer (Promega Biotec, Madison, WI).

Immunofluorescence Microscopy

Erythroid cells from chicken were prepared and processed for immunofluorescence microscopy essentially as described by Murphy et al. (1986), with the following modifications: cells obtained from whole blood or bone marrow were extracted with 0.2% Triton X-100 in microtubule stabilizing buffer (Osborn and Weber, 1982) for 15 s and treated first with 0.5% formaldehyde for 20 min and then with methanol at −20°C for 6 min. After rehydration with 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.3, cells were labeled with monoclonal antibodies to α- and β-tubulin (Amersham Corp., Arlington Heights, IL) and rabbit antibodies to syncolin from chicken erythrocytes, or to hog brain MAP 2, followed by fluorescein-labeled goat anti-mouse (Dianova, Hamburg, Germany) and Texas Red–labeled donkey anti-rabbit (Amersham Corp.) antibodies. For the reversible depolymerization of marginal band microtubules, erythrocytes resuspended in PBS containing 25 mM glucose were incubated as indicated and processed for microscopy as above, except that fixation with formaldehyde was omitted. Specimens were viewed in an Axiophot photomicroscope using a 100× Plan-Neofluar oil immersion objective (both from Carl Zeiss). Photographs were taken using Ilford FP4 35-mm film developed at 200 ASA or Ilford HP5 35-mm film developed at 800 ASA.

Electron Microscopy

Uranyl acetate staining was performed on ultraviolet-irradiated, 400-mesh copper grids (Polaron, Watford, UK) coated with Formvar. Protein samples were adsorbed onto the grids for 1 min, followed by fixation with 1% glutaraldehyde and staining with 1.25% aqueous uranyl acetate for 1 min. For rotary and unidirectional shadowing, protein samples were treated according to the protocol of Tyler and Branton (1980), with slight modifications (Foisner and Wiche, 1987). Immunoelectron microscopy was done essentially as described by Foisner and Wiche (1985). Adjustments to the use of chicken erythrocytes were as follows: Extraction and fixation of the cells were performed in 10 mM MES, pH 6.1, 137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.5 mM glucose, 2 mM $MgCl_2$, 2 mM EGTA, and 4 mM $NaHCO_3$. Furthermore, the incubations of the speci-
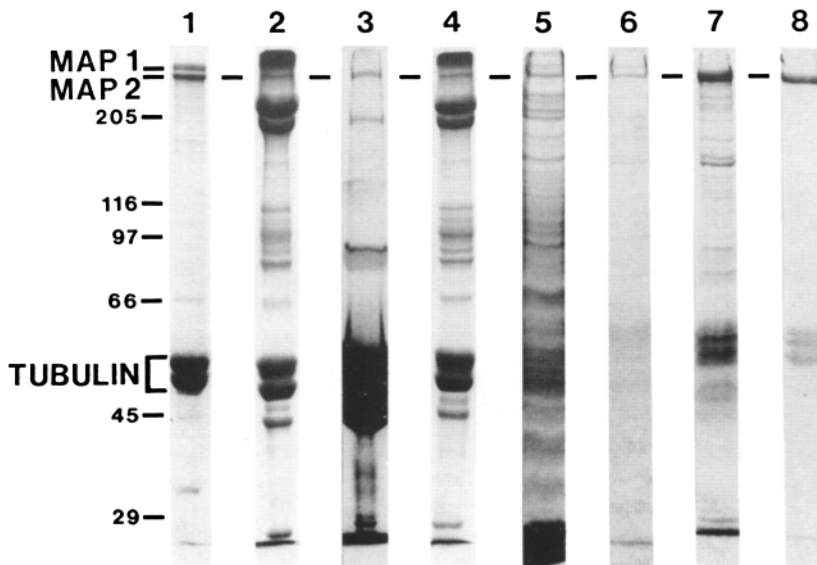
Figure 1. Purification of syncolin monitored by SDS-PAGE. Lane 1, standard (twice-cycled microtubule proteins from hog brain); lane 2, pellet of chicken erythrocyte microtubules obtained after one cycle of temperature-dependent assembly; lane 3, pellet of chicken erythrocyte microtubules obtained after two cycles of temperature-dependent assembly; lane 4, pellet of erythrocyte microtubules polymerized by taxol; lane 5, proteins released from taxol-polymerized microtubules by incubation at 0°C; lane 6, syncolin-containing fraction of sucrose density centrifugation; lane 7, hydroxyapatite-bound fraction eluted with 300 mM phosphate; lane 8, DEAE-cellulose-purified syncolin fraction. Note, that different amounts of protein were loaded onto the gel in lanes 1–8 in order to visualize also minor components in some of the fractions. Numbers ($\times 10^{-3}$) indicate, from top to bottom, the molecular weight standards myosin, β-galactosidase, phosphorylase b, BSA, ovalbumin, and carbonic anhydrase.

mens with antibodies were carried out in 20 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EGTA, 2 mM MgCl$_2$, and 10% FCS. Cells were incubated with polyclonal rabbit antibodies to syncolin, monoclonal antibodies to tubulin, or a mixture of both; the secondary immunoreagents (Jansen Pharmaceutica, Beerse, Belgium) were goat anti–rabbit IgG linked to 10-nm gold particles and goat anti–mouse IgG conjugated to 5-nm gold particles. Specimens were viewed in a 100C electron microscope (JEOL) operated at 80 kV.

Other Procedures

For cosedimentation of syncolin or MAP 2 with polymerized microtubules, the proteins were incubated with phosphocellulose-purified (Weingarten et al., 1975) hog brain or chicken erythrocyte tubulin at indicated amounts in 25 mM MES, pH 6.7, 1 mM EGTA, 1 mM MgCl$_2$, and 20 μM taxol (buffer E) at 37°C for 20 min. These mixtures were then centrifuged through a cushion of 15% sucrose in buffer E at 15,000 g and 25°C for 45 min. The supernatants were collected and the pellets were washed with prewarmed buffer E and centrifuged as above. Washed pellets and original supernatants were analyzed by SDS-PAGE and densitometric scanning of electropherograms using tubulin and syncolin samples of known concentrations as standards.

Microtubule formation was monitored by measurement of the change in turbidity at 350 nm using a spectrophotometer (Beckman Instruments, Inc.) as described (Williams and Detrich, 1979). Protein was determined according to the method of Bradford (1976) using BSA as the standard.

shown). Attempts to separate this protein from tubulin using techniques known to work for mammalian brain MAP 2, such as heat treatment or phosphocellulose chromatography, failed.

A separation of syncolin from tubulin was achieved, however, using preparations of erythrocyte microtubules that were assembled by taxol (Vallee, 1982) instead of temperature-dependent polymerization cycles. The protein composition of microtubules isolated by taxol-driven assembly (Fig. 1, lane 4) was very similar to that of microtubules obtained by one round of temperature-dependent assembly (Fig. 1, lane 2). Syncolin was released from taxol-stabilized microtubules simply by resuspending the pellet in ice-cold buffer B. Among other proteins recovered were hemoglobin, as the main component, and two high molecular weight proteins comigrating with hog brain MAP 1 (Fig. 1, lane 5).

To further purify syncolin, the protein fraction released from microtubules was first subjected to sucrose density centrifugation. Syncolin sedimented away from the bulk of the other proteins at 22–27S (Fig. 1, lane 6). Subsequent chromatography of these fractions on hydroxyapatite columns

Results

Purification of Syncolin from Chicken Erythrocytes

Microtubules prepared by two rounds of temperature-dependent in vitro assembly (Karr et al., 1979) from soluble extracts of chicken erythrocytes consisted of >90% tubulin, three major proteins of apparent M_r 280,000 (syncolin), 200,000, and 100,000, and several low molecular weight proteins including hemoglobin (Fig. 1, lane 3). In comparison, hog brain microtubules prepared in a similar way contained, aside from tubulin, predominantly the high molecular weight proteins MAP 1 and MAP 2 (Fig. 1, lane 1). Syncolin contained in preparations of chicken erythrocyte microtubules was immunoreactive with antibodies to hog brain MAP 2, but not with antibodies to hog brain MAP 1 (Wiche et al., 1983), as revealed by immunoblotting (data not
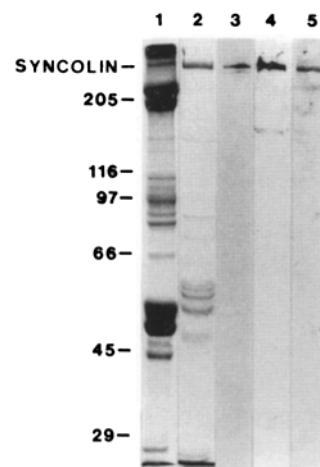

Figure 2. Coomassie blue staining (lanes 1 and 2) and immunoblotting of erythrocyte proteins using rabbit antibodies to hog brain MAP 2 (lanes 3 and 4) and to syncolin (lane 5). Lanes 1 and 3, pellet of taxol-polymerized erythrocyte microtubules; lanes 2, 4, and 5, hydroxyapatite-purified syncolin. Note that anti–MAP 2 antibodies affinity-purified by either method a or b (see text) yielded identical results. Molecular weight standards are as in Fig. 1.
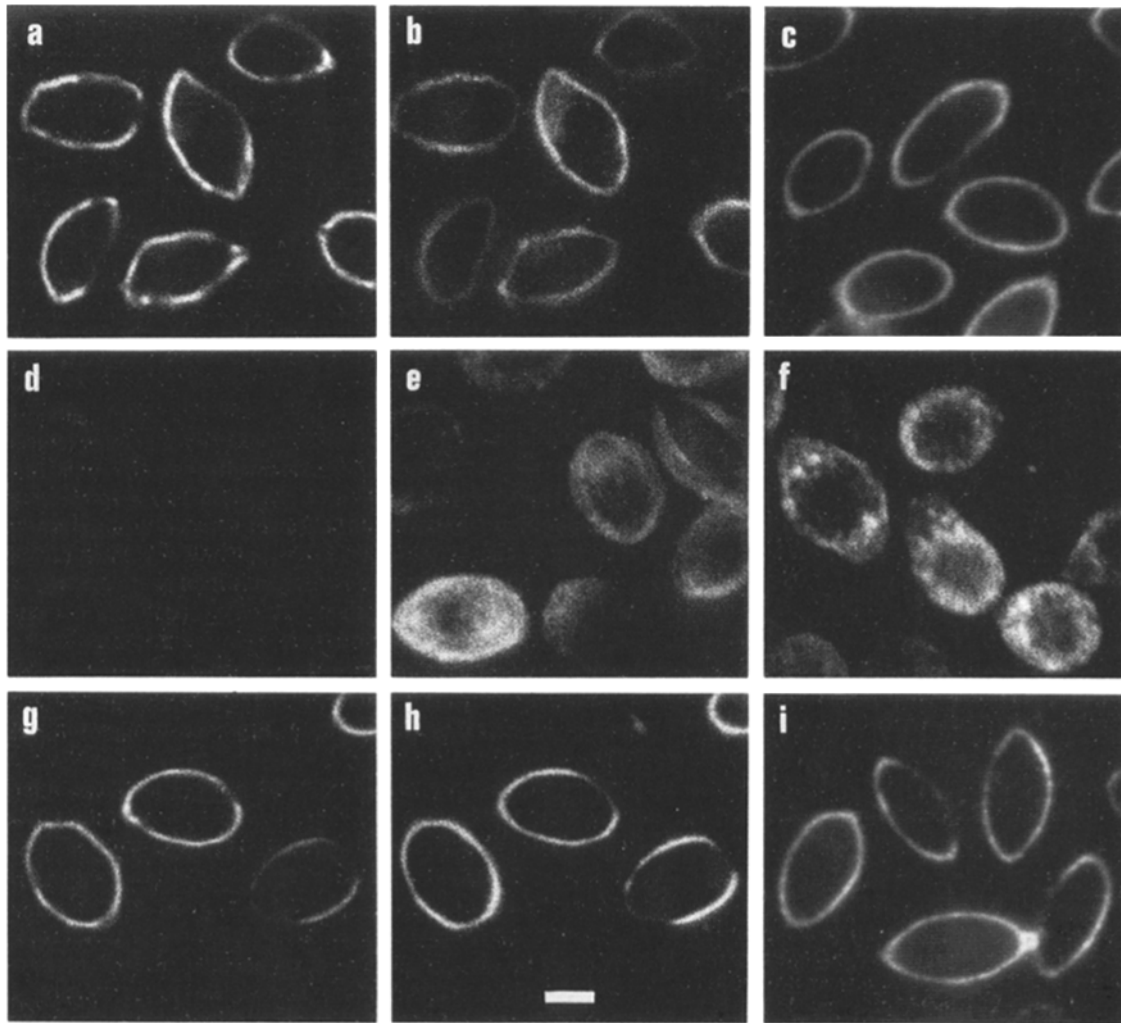
Figure 3. Double (anti-tubulin/anti-syncolin) and anti-MAP 2 immunofluorescence microscopy of mature chicken erythrocytes. *a*, *d*, and *g*, monoclonal antibodies to tubulin; *b*, *e*, and *h*, rabbit antibodies to syncolin; and *c*, *f*, and *i*, rabbit antibodies to hog brain MAP 2. Erythrocytes incubated at 39°C for 60 min (*a–c*); at 0°C for 60 min (*d–f*); or first at 0°C for 60 min and then at 39°C for 60 min (*g–i*). After preincubations cells were processed for immunofluorescence microscopy as described in the text. Bar, 5 μm.

and selective elution with 300 mM phosphate yielded fractions highly enriched in syncolin and a set of proteins of 50,000–65,000 M_r (Fig. 1, lane *7*). In a final step of DEAE-cellulose chromatography most of the remaining contaminating proteins were adsorbed to the column at 200 mM phosphate, while syncolin and part of the proteins of 50,000–65,000 M_r eluted under these conditions with the void volume (Fig. 1, lane *8*). The overall yield of syncolin in this fraction was between 1 and 2% of its total.

Attempts aimed at separating syncolin from the lower molecular weight proteins using gel filtration or other chromatographic methods were unsuccessful. We conclude, therefore, that the 50,000–65,000-M_r proteins were tightly associated with syncolin. Although the sizes of these proteins were in the molecular weight range of tau proteins, known to be present in chicken erythrocytes (Murphy and Wallis, 1985), two observations spoke against them being tau proteins. First, antibodies to hog brain tau proteins shown to be crossreactive with their erythrocyte counterparts, showed no reaction with the proteins of 50,000–65,000 M_r.

Second, after a heat treatment of the proteins released from taxol-polymerized microtubules tau proteins remained soluble, while most of the 50,000–65,000-M_r proteins, like syncolin itself, were heat irresistant (unpublished data).

The analysis of cell fractions obtained at various stages of purification by SDS-PAGE and densitometric scanning of electropherograms revealed that about two-thirds of erythrocyte syncolin was soluble and about half of this fraction cosedimented with taxol-polymerized microtubules. The amount of syncolin per cell was estimated to be 0.05% of the total protein. In conjunction with the amount of tubulin (0.45% of total protein), this yielded a molar ratio of 1:23 for syncolin versus tubulin dimers.

At all stages of purification chicken erythrocyte syncolin maintained its immunoreactivity toward antibodies to hog brain MAP 2. In taxol-assembled microtubule preparations a doublet of closely spaced immunoreactive bands comigrating with brain MAP 2 was detectable (Fig. 2, lane *3*). However, in fractions enriched in syncolin only the major (upper) band of this doublet and an additional minor 160-kD band
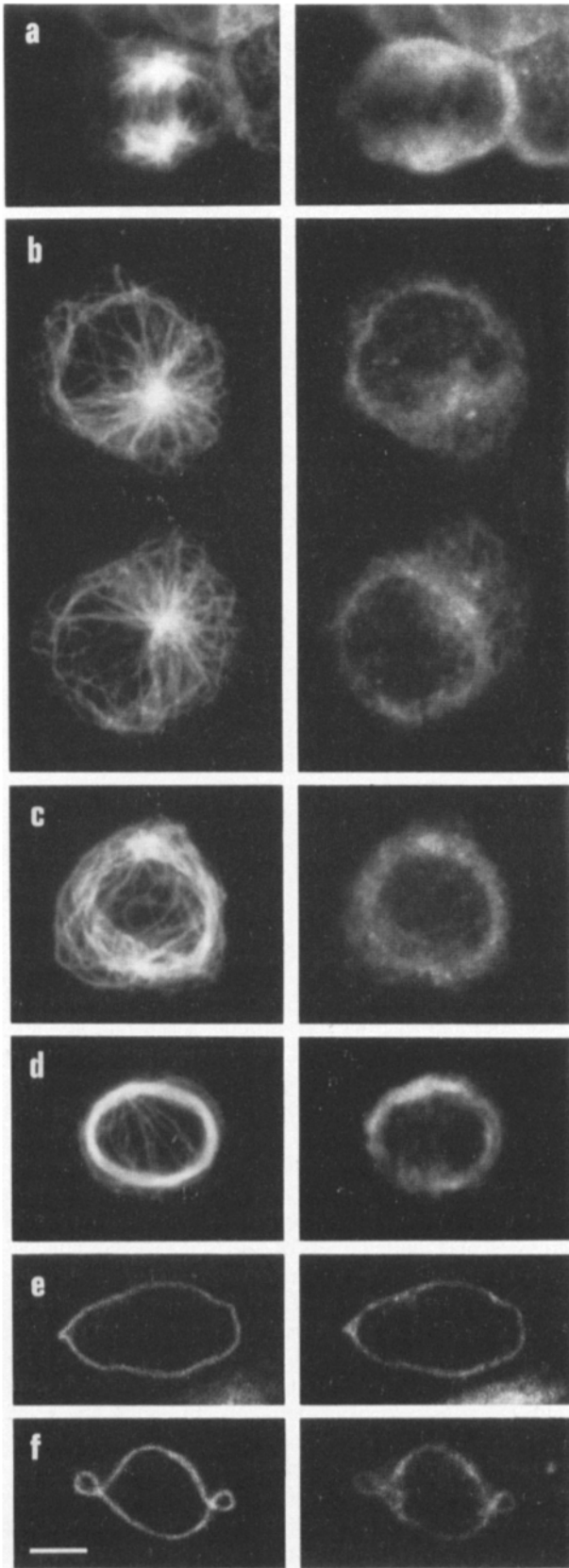
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Figure 4. Double immunofluorescence microscopy of developing erythrocytes and mature erythrocytes using monoclonal antibodies to tubulin (*left column*) and affinity-purified rabbit antibodies to syncolin (*right column*). Chicken bone marrow cells were processed for immunofluorescence microscopy as described in the

were detected (Fig. 2, lane *4*). It is likely that both lower molecular weight bands represented degradation products of syncolin arising during purification. Antibodies raised by immunization of rabbits with electrophoretically purified chicken erythrocyte syncolin showed the expected cross-reactivity with syncolin (Fig. 2, lane *5*). They were not reactive, however, with hog or chicken brain MAP 2 (data not shown).

Immunolocalization of Syncolin in Mature Chicken Erythrocytes and Developing Erythroblasts

To localize syncolin in relation to microtubules in mature chicken erythrocytes, double immunofluorescence microscopy was performed using affinity-purified antibodies to syncolin and monoclonal antibodies to α- and β-tubulin; in addition, cells were stained with antibodies to hog brain MAP 2. In erythrocytes kept at 39°C to preserve marginal band microtubules, the staining patterns observed in all cases were very similar (Fig. 3, *a–c*). In general, however, the marginal bands were more sharply outlined using antibodies to tubulin than with the other antibodies, probably because of a higher density of epitopes. In cells preincubated at 0°C to depolymerize microtubules, no anti-tubulin staining was observed (Fig. 3 *d*), whereas diffuse anti-syncolin as well as anti–MAP 2 staining was found throughout the cytoplasm (Fig. 3, *e* and *f*). Thus, it is likely that after the depolymerization of microtubules syncolin bound to other cellular components, with an affinity not affected by our fixation protocol (0.2% Triton X-100 followed by methanol). In cells preincubated first at 0 and then at 39°C to disassemble and reform the marginal band, the staining patterns of all antibodies were indistinguishable from those of cells always kept at 39°C (Fig. 3, *g–i*). This indicated that the association of syncolin with marginal band microtubules was reversible.

To study the distribution of syncolin in the course of marginal band formation in differentiating erythroblasts, dispersed bone marrow cells from the long bones of 1-wk-old chicks were examined by double immunofluorescence microscopy as above. As shown in Fig. 4, syncolin was detectable in cells at all stages of differentiation, including erythrocyte precursor cells, which were identified as cells with a large nucleus and prominent nucleoli by phase contrast microscopy. In the early stages of development syncolin showed a distribution distinct from that of the microtubules. In dividing erythroblasts the peripheral part of the cytoplasm and the mitotic spindle poles were diffusely stained by antibodies to syncolin (Fig. 4 *a*), and in early erythroblasts diffuse anti-syncolin staining was found throughout the cell (Fig. 4 *b*). In the latter case, however, anti-syncolin staining was brightest in areas where microtubules were more abundant, such as the perinuclear region and the centrosome (Fig. 4 *b*). In polychromatophilic erythroblasts, where the mar-

text. (*a*) Dividing erythroblast; (*b*) early erythroblasts with centrosomal microtubules; (*c*) polychromatophilic erythroblast with microtubules attached to centrosomes at the perimeter of the cell; (*d*) polychromatophilic erythroblast with bundled microtubules; (*e*) mature erythrocyte with marginal band of normal morphology; (*f*) mature erythrocyte with supertwisted marginal band. Bar, 5 μm.
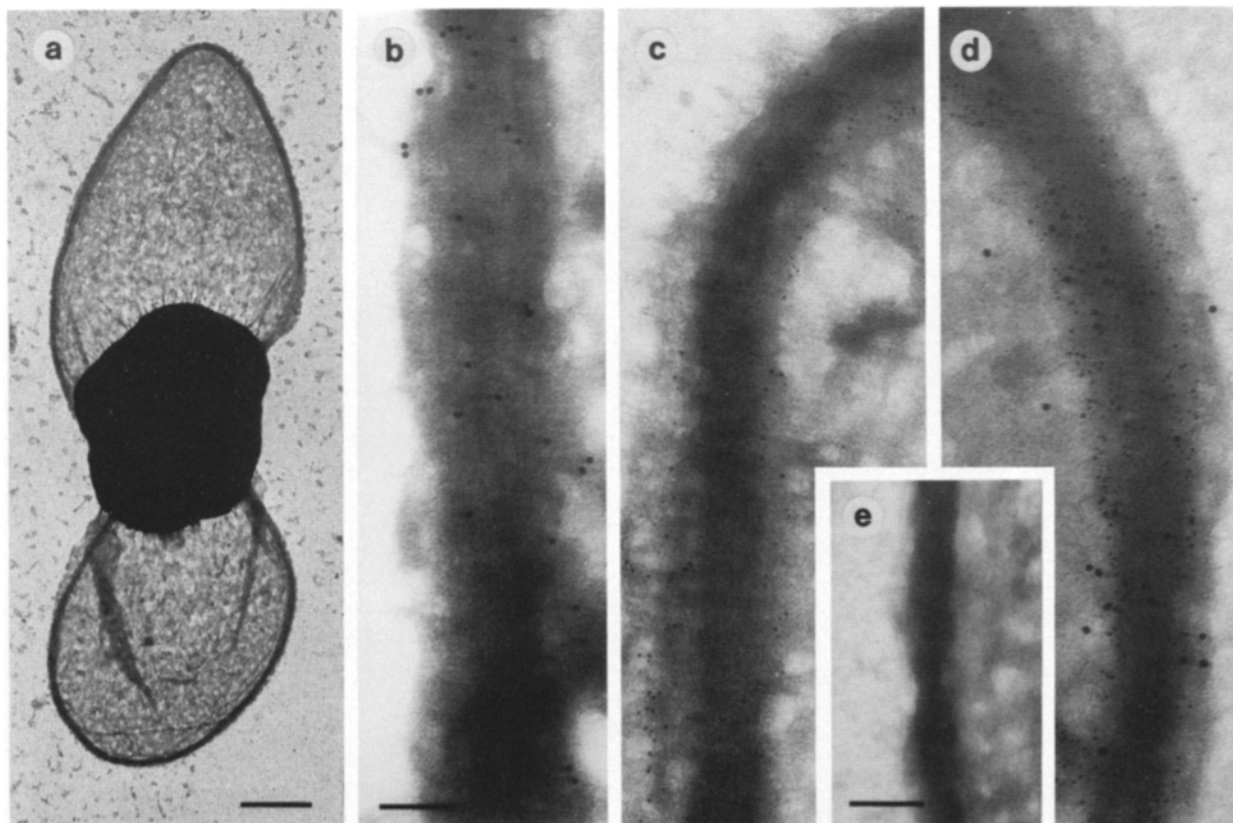
Figure 5. Whole-mount immunoelectron microscopy of mature chicken erythrocytes. Chicken erythrocytes were processed for immunoelectron microscopy as described in the text. (*a*) Mature erythrocyte extracted with Triton X-100 (unlabeled); (*b–d*) immunogold labeling of marginal bands using as primary immunoreagents rabbit antibodies to syncolin (*b*), mouse antibodies to tubulin (*c*), or a mixture of both antibodies (*d*); (*e*) cells incubated with secondary immunoreagents alone. Bars: 1 μm (*a*); 100 nm (*b–d*); 167 nm (*e*).

ginal band started to form, anti-syncolin positive dotlike structures were found largely in the region of the marginal band, with additional staining throughout the cytoplasm (Fig. 4, *c* and *d*). Finally, in mature erythrocytes the only anti-syncolin positive structure observed colocalized with the marginal band (Fig. 4 *e*). Such colocalization was observed also in cases of supertwisted marginal bands (Fig. 4 *f*). These data suggested that syncolin was already present in cells before microtubules became bundled. Whether its confinement to the marginal band followed or preceded the organization of tubulin remains to be shown.

Immunoelectron Microscopy of Marginal Band Syncolin

After extraction of mature chicken erythrocytes with 0.1% Triton X-100 and fixation with 1% glutaraldehyde, the marginal band was visualized in the electron microscope as a highly compact bundle (Fig. 5 *a*). As revealed by the immunogold labeling technique, such bundles were immunoreactive with antibodies to both tubulin (Fig. 5, *c* and *d*) and syncolin (Fig. 5, *b* and *d*). The less intense labeling patterns observed with antibodies to syncolin compared with anti-tubulin antibodies may have been due to the relatively lower content of syncolin in the marginal band, to limited accessibility of syncolin epitopes within the bundled structure, or to both. Hardly any gold label was detectable in control experiments carried out without primary antibodies (Fig. 5 *e*).

In Vitro Interaction of Syncolin with Microtubules

To examine whether syncolin bound to in vitro polymerized microtubules and whether such binding was saturable, mixtures of increasing amounts of the protein with a constant amount of taxol-stabilized hog brain microtubules were sedimented through a cushion of sucrose, and pellets as well as supernatants were analyzed by SDS-PAGE and densitometric scanning of electropherograms. As shown in Fig. 6, syncolin bound to tubulin in a linear relationship up to a concentration of ∼150 nM, equivalent to a molar ratio of syncolin versus tubulin dimers of ∼1:12.5; at higher concentrations the binding curve leveled off sharply, indicating saturation. Similar ratios (1:12.6) of cosedimenting proteins were obtained in analogous experiments performed with syncolin and taxol-polymerized microtubules from chicken erythrocytes (Fig. 7, lanes *1* and *2*). Compatible ratios (1:11.7) were also observed in control experiments, in which hog brain MAP 2 was used instead of syncolin (Fig. 7, lanes *3* and *4*). In contrast, two unrelated proteins, BSA and creatine kinase, were not sedimented at all (not shown).

As shown in Fig. 8, the amount of syncolin cosedimenting with hog brain or chicken erythrocyte microtubules decreased as the concentration of salt in the reaction mixture was increased, whereas the level of sedimented tubulin remained nearly constant over the same concentration range. Syncolin was displaced from erythrocyte microtubules at lower concentrations of salt than from brain microtubules, indicating that its binding to the former was weaker.
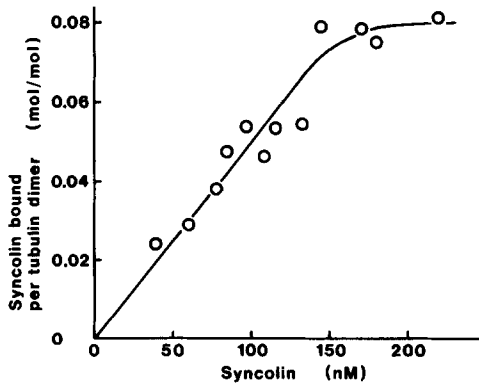
Figure 6. Saturable binding of syncolin to taxol-polymerized microtubules. Constant amounts of hog brain tubulin (0.2 mg/ml) and increasing amounts of purified syncolin (0–0.06 mg/ml) were incubated in buffer E at 37°C for 20 min. Mixtures were then centrifuged and fractions were analyzed as described in the text.

To assess whether syncolin, like other MAPs, promoted the polymerization of microtubules, hog brain tubulin (1.1 mg/ml) was incubated with syncolin or, as a control, hog brain MAP 2, both at concentrations of 0.06–0.2 mg/ml, and microtubule assembly at 37°C was monitored by turbidity measurements. Unlike MAP 2, syncolin did not cause a steady increase of turbidity, characteristic of MAP-promoted microtubule assembly. Instead, a saltatory turbidity increase that was dependent on the amounts of syncolin and tubulin, but independent of GTP and temperature, was observed (data not shown).

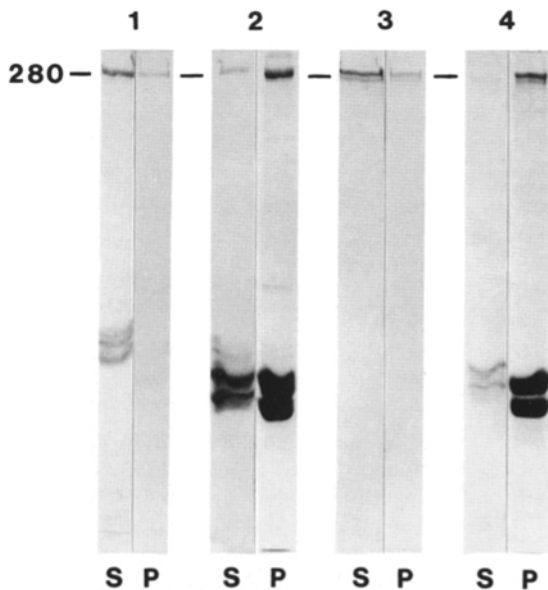The electron microscopy of chicken erythrocyte or hog

Figure 7. Cosedimentation of syncolin and MAP 2 with taxol-polymerized erythrocyte microtubules. Syncolin and hog brain MAP 2, each at a concentration of 0.08 mg/ml, were incubated with 0.16 mg/ml of erythrocyte tubulin in buffer B at 37°C for 15 min. After centrifugation (see text) equal amounts of the pellets (P) and supernatants (S) were analyzed by SDS-PAGE. Lanes 1, syncolin alone; lanes 2, syncolin and tubulin; lanes 3, MAP 2 alone; lanes 4, MAP 2 and tubulin. Number, $M_r \times 10^{-3}$.
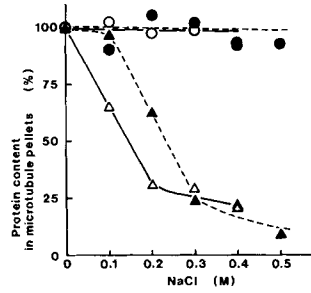

Figure 8. Effect of ionic strength on syncolin binding to taxol-polymerized microtubules. Syncolin (0.06 mg/ml) and tubulin from hog brain or from chicken erythrocytes both at 0.2 mg/ml were incubated in buffer E at 37°C for 10 min. After addition of sodium chloride at concentrations indicated, incubations at 37°C were continued for 10 min, and samples were then centrifuged and analyzed as described in the text. Open symbols, syncolin (△) and chicken erythrocyte tubulin (○); closed symbols, syncolin (▲) and hog brain tubulin (●).

brain microtubules, both sedimented in the absence of syncolin and then resuspended and stained with uranyl acetate, revealed largely single entities of typical ultrastructure (Fig. 9 a, and data not shown). Microtubules of both preparations were aggregated into bundles, however, when preincubated with syncolin before their sedimentation (Fig. 9, b–d). Such bundling was observed at molar ratios of syncolin versus tubulin dimers as low as 1:74 (Fig. 10). In this case, about two-thirds of the microtubules were found in bundles containing two to six microtubules. With increasing proportions of syncolin to tubulin dimers, the bundles formed became larger, and at a ratio of 1:20 >90% of the tubulin was found in bundles of 4–15 microtubules. Bundled microtubules appeared to be crosslinked via globular surface-attached structures (Fig. 9, c and d). A few single microtubules extending from the bundles were in part bare of surface structures (Fig. 9 c, arrowheads). Erythrocyte and brain microtubules incubated with hog brain MAP 2 formed no bundles, but were randomly dispersed. Similarly, no bundles were observed when mixtures of microtubules and syncolin were sedimented in the presence of 400 mM sodium chloride (Fig. 10).

Ultrastructure of Syncolin

The molecular shape of purified syncolin was examined by electron microscopy of uranyl acetate–stained and rotary or unidirectionally shadowed specimens. Uranyl acetate staining (Fig. 11, a and b) revealed a ring- or spherelike structure of syncolin with a diameter of 13 nm and a central negative stain-filled cavity or channel. The possibility that these structures represented microtubule rings could be ruled out because of (a) their considerably smaller size and (b) the absence of any detectable tubulin in the samples tested. Rotary and unidirectionally shadowed syncolin appeared as a globular structure of 30 nm diameter (Fig. 11, c and d). In contrast, molecules of hog brain MAP 2, a typically filamentous MAP, were visualized by rotary and unidirectional shadowing as long, thin, and flexible structures with knobs at one or both ends (Fig. 11, e and f). The lengths of these structures varied between 120 and 200 nm, in fair agreement with previously reported measurements of up to 185 nm (Voter and Erickson, 1982) and 90 ± 30 nm (Gottlieb and Murphy, 1985).

Assuming a spherical shape with an average diameter of 13 nm, and without taking into account the cavity or channel indicated by uranyl acetate staining, the molecular weight of
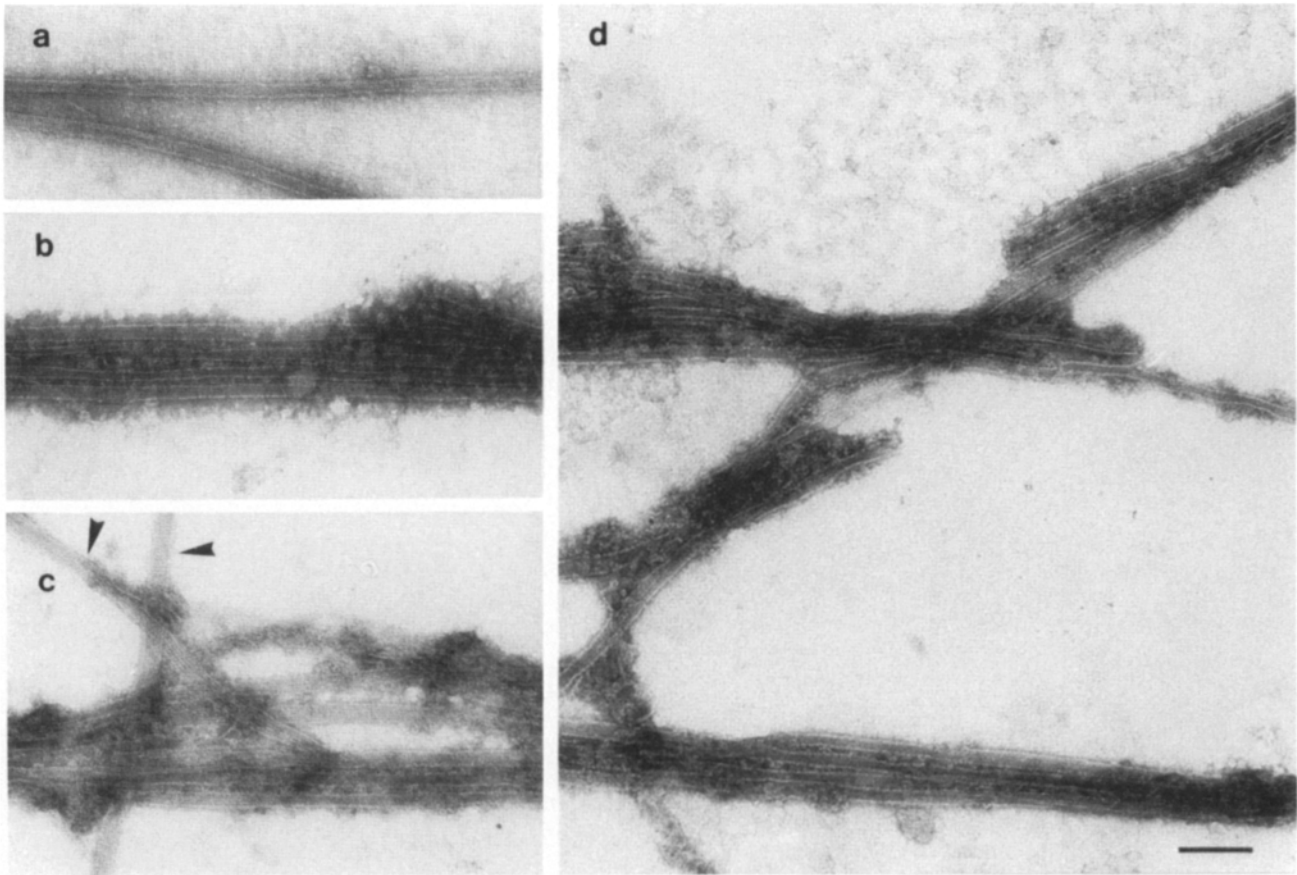
Figure 9. Electron microscopy of uranyl acetate–stained microtubules reconstituted in vitro. Phosphocellulose-purified tubulin preparations from erythrocytes or brain were incubated with purified syncolin in the presence of taxol and sedimented through sucrose as described in the text. Pellets resuspended in buffer B without taxol and GTP were processed for electron microscopy. (*a*) Erythrocyte microtubules polymerized in the absence of syncolin; (*b* and *c*) erythrocyte microtubules polymerized in the presence of syncolin; (*d*) brain microtubules polymerized in the presence of syncolin. Note that microtubules without detectable crosslinking structures were not bundled (*arrowheads* in *c*). Bar, 100 nm.

syncolin was calculated as 960,000. This was in good agreement with a value of 1,020,000 determined by gel permeation chromatography on Sepharose CL-4B (data not shown). Taking into account syncolin's average molecular weight of 990,000, a sedimentation coefficient of 22–27S, and an average partial specific volume of 0.72 ml/g (Cantor and Schimmel, 1980), a frictional coefficient of $f = 1.7$–2.1×10^{-7} g/s was estimated. The theoretical frictional coefficient (f_{min}) of a 990,000-mol-wt spherical molecule would be 1.24×10^{-7} g/s (Cantor and Schimmel, 1980). This yields a f/f_{min} ratio of 1.4 to 1.7, indicating that syncolin molecules have a nearly spherical shape in solution; molecules with elongated shapes, like laminin or plectin, have considerably higher f/f_{min} ratios (Engel et al., 1981; Foisner and Wiche, 1987). Consistent with this, a value of 0.25–0.5 was obtained for syncolin's axial ratio, under the assumption that hydration of syncolin molecules occurred as a uniform shell with 0.3–0.5 g of solvent per g protein (Cantor and Schimmel, 1980; Van Holde, 1971).

Based on syncolin's frictional coefficient of 1.7–2.1×10^{-7} g/s, a Stokes radius of 9.1–11.1 nm was calculated. This was in fair agreement with a value of 12.2 nm, determined by gel permeation chromatography on Sepharose CL-4B (data not shown).

Discussion

In this study we described the isolation of syncolin, a chicken erythrocyte 280,000-mol-wt protein, whose most distinctive features characterized were the colocalization with marginal band microtubules and its ability to bundle microtubules in vitro. The specificity of syncolin's binding to microtubules was demonstrated in two ways. First, syncolin cosedimented with taxol-polymerized tubulin in a saturable manner, indicating the presence of a limited number of binding sites. Second, its binding to microtubules was salt sensitive, excluding the possibility of nonspecific trapping of the relatively large syncolin molecules by sedimented tubulin polymers. Furthermore, the following observations provided strong evidence that syncolin was effecting the bundling of microtubules: the number of microtubules per bundle was dependent on the amount of syncolin cosedimenting with microtubules, and microtubules stripped of syncolin by salt consisted mainly of single polymers. Syncolin's bundling activity became noticeable already at low ratios of syncolin versus tubulin dimers, such as 1:144, where 20% of the total microtubule population was observed in the form of small bundles consisting of two to three microtubules. The higher the amount of syncolin bound to microtubules, the larger the
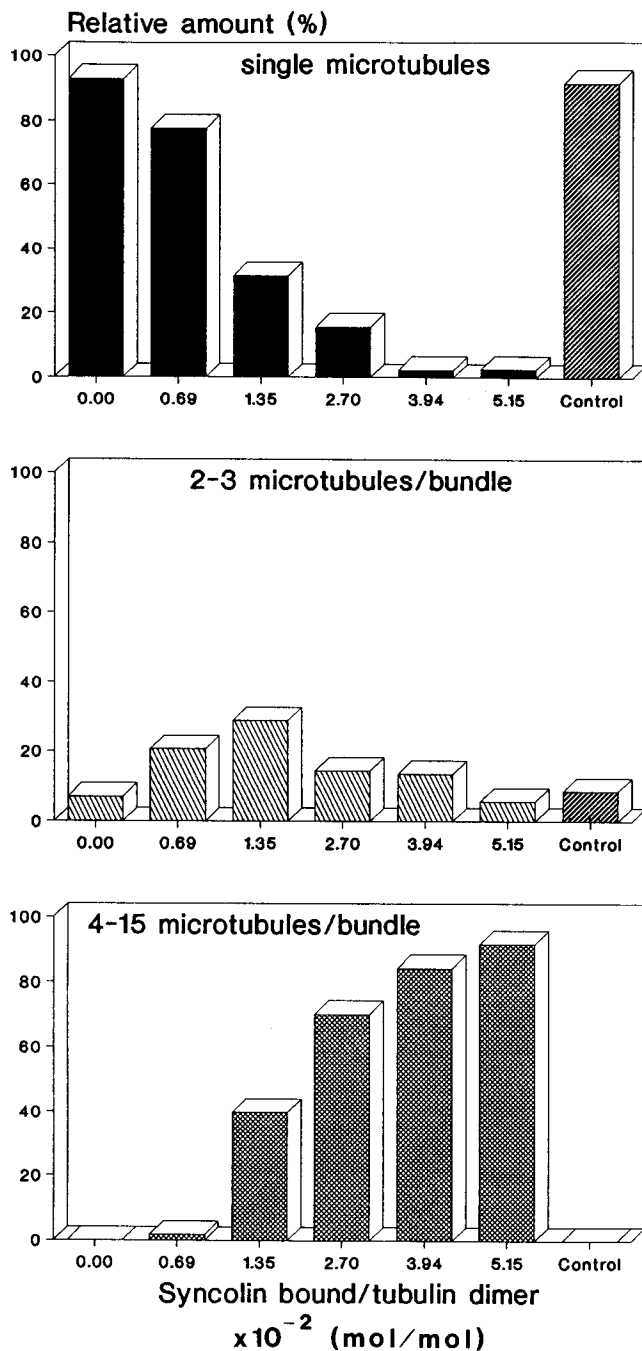
Relative amount (%)

Figure 10. Effect of syncolin on the formation of microtubule bundles. Constant amounts of taxol-polymerized hog brain tubulin (0.25 mg/ml) and increasing amounts of purified syncolin (0–40 μg/ml) were incubated in buffer E at 37°C for 20 min. As a control, taxol-polymerized tubulin (0.25 mg/ml) and syncolin (30 μg/ml) were incubated in buffer E in the presence of 400 mM NaCl. Mixtures were then centrifuged and analyzed as described in the text. Aliquots of the pellets resuspended in buffer E were processed for electron microscopy as described. To estimate their statistical distribution ~250 microtubules were counted in a randomly selected field of each grid. Bundles containing two to three microtubules were considered as intermediates in the formation of larger bundles because they were observed already at very low syncolin concentrations, but never made up >30% of the total microtubule population.

bundles became. At a ratio of syncolin versus tubulin dimers of 1:20, which is close to the calculated in vivo value, about one-third of the microtubules was found in bundles containing 10–15 microtubules. This is the number observed in mature chicken erythrocytes (Goniakowska-Witalinska and Witalinski, 1976). Thus, syncolin can be considered as a potent microtubule bundling activity.

Additional evidence for syncolin molecules mediating microtubule bundle formation was provided by the following: (a) the space between coaligned microtubules was ~10 nm, about the size of syncolin molecules; (b) globular structures attached to the surfaces of coaligned microtubules were indeed observed when syncolin was pelleted with taxol-polymerized microtubules; and (c) when gold-immunoelectron microscopy was performed on syncolin cosedimented with microtubules, the gold label was found along the bundled microtubules (data not shown). Finally, it should be noted that the compact microtubule bundle observed in vitro after uranyl acetate staining corresponded well to a similarly compact microtubule bundle visualized after similar staining in mature nucleated erythrocytes such as from newt (Sloboda and Dickersin, 1980) and chicken (Murphy and Wallis, 1983b).

In the early stages of erythrocyte development, syncolin was distributed distinctly from microtubules and, hence, apparently was associated with other structures. However, when the marginal band began to form, syncolin's distribution was observed to shift to the region of the developing band. Thus, the temporal and spatial correlation of microtubule bundle formation and association of syncolin with marginal band microtubules strongly supports the notion that syncolin plays a role in the formation of the marginal band. Whether syncolin's association with microtubules is a primary cause for bundle formation or whether the protein serves a secondary function, such as stabilization of an already formed marginal band, remains to be clarified.

Two sorts of proteins have been suggested (Birgbauer and Solomon, 1989) to be associated with marginal band microtubules: one that binds exclusively to tubulin and mediates microtubule–microtubule interactions (Sloboda and Dickersin, 1980; Centonze et al., 1985), and another that defines the position of the marginal band and/or stabilizes it by anchoring bundled microtubules into the plasma membrane or linking them to other cytoskeletal elements (Kim et al., 1987; Birgbauer and Solomon, 1989). Syncolin may belong to the first sort of marginal band–associated proteins for the following reasons. First, whenever supertwisted marginal bands were observed, syncolin colocalized with microtubules, but not with the plasma membrane. Second, when marginal band microtubules were disassembled by cold treatment, anti-syncolin staining became diffuse, but the staining reappeared at the marginal band upon microtubule reformation at 39°C.

Based on the available evidence, we consider it likely that syncolin corresponds to a MAP 2–related protein that previously has been shown to be a component in the marginal band of amphibian and avian erythrocytes (Sloboda and Dickersin, 1980; Centonze et al., 1985; Centonze and Sloboda, 1986). However, the data obtained in this study indicate that syncolin's relationship to MAP 2 is restricted to comigration in SDS-PAGE and to one or a few common epitopes. The proteins were found to be strikingly different with
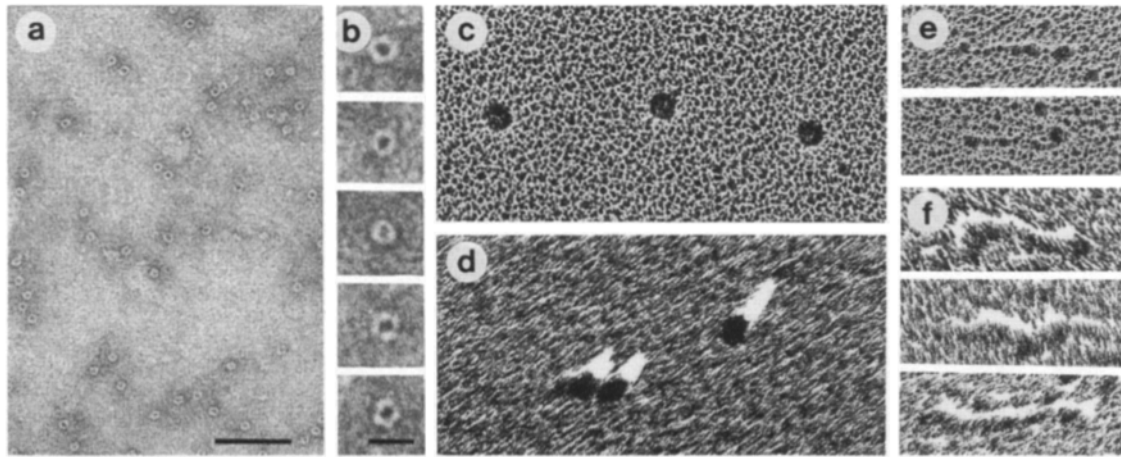
Figure 11. Electron microscopy of purified syncolin and hog brain MAP 2. (*a–d*) Syncolin; (*e* and *f*) MAP 2. (*a* and *b*) Uranyl acetate staining; (*c* and *e*) rotary shadowing; (*d* and *f*) unidirectional shadowing. Bars: 20 nm (*b*); 100 nm (*a* and *c–f*).

regard to molecular structure, as revealed on the ultrastructural level, and to a number of biochemical characteristics.

In conclusion, the isolation of syncolin, whose proposed specialized function is the bundling of microtubules in mature (chicken) erythrocytes, provides a promising opportunity for further studies on the structure–function relationship of non-neuronal MAP species on the biochemical and genetic level.

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