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Gamma-Tubulin in Chicken Erythrocytes: Changes in Localization During Cell Differentiation and Characterization of Cytoplasmic Complexes

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ABSTRACT **The mechanism of marginal band (MB) formation in differentiating erythroid cells is not fully understood, and the proteins involved in nucleation of MB microtubules are largely unknown. To gain insights into the function of γ-tubulin in MB formation, we have followed its distribution in developing chicken erythrocytes and characterized soluble forms of the protein. In early stages of erythroid cells differentiation, γ-tubulin was present in microtubule-organizing centers, mitotic spindles, as well as on MB. Its subcellular localization changed in the course of differentiation, and in postnatal peripheral erythrocytes γ-tubulin was found only in soluble forms. After cold-induced depolymerization γ-tubulin in erythroid cells formed large clusters that were not observed in matured cells, and re-growth experiments demonstrated that γ-tubulin was not present in distinct nucleation structures at the cell periphery. Soluble γ-tubulin formed complexes of various size and large complexes were prone to dissociation in the presence of high salt concentration. Interaction of γ-tubulin with tubulin dimers was revealed by precipitation experiments. γ-Tubulin occurred in multiple charge variants whose number increased in the course of erythrocyte differentiation and corresponded with decreased binding to MB. The presented data demonstrate for the first time that γ-tubulin is a substrate for developmentally regulated posttranslational modifications and that the binding properties of γ-tubulin or its complexes change during differentiation events.**

Key words: chick embryo; marginal band; nucleated erythrocytes; microtubules; γ-tubulin

INTRODUCTION

Microtubules, assembled from α- and β-tubulin heterodimer, play a decisive role for many cellular functions such as intracellular organization, ordered vesicle transport and cell division, to name but a few. Microtubules exhibit an inherent property of dynamic instability (Mitchison and Kirschner, 1984), and this property is crucial for microtubule functions and their plasticity. In some terminally differentiated cell types such as neurons (Baas and Black, 1990), myocytes (Tassin et al., 1985), polarized epithelial cells (Bre et al., 1990), platelets (Kenney and Linck, 1985), and nucleated erythrocytes of nonmammalian vertebrates (Behnke, 1970) there exist, however, highly stable microtubule configurations. Although the mechanisms and functional significance of dynamic instability are known in great detail, mechanisms governing the conversion of dynamic microtubules into highly ordered structures remain poorly understood.

In mature chicken erythrocytes, the marginal band (MB) consists of a highly ordered and stable microtubule bundle that is located under the plasma membrane at the equatorial plane of the cells (Behnke, 1970; Cohen, 1991). The MB helps maintain the elliptical shape of the cells and helps resist the deformation forces to which the cells are exposed during circulation. Formation of the MB of microtubules represents the most striking event during differentiation of avian spherical erythroblasts to immature flattened discoids and finally to mature ellipsoidal erythrocytes. Changes in shape and size of the nucleus and rearrangements of the cytoskeleton are characteristic for this process. Thus, the formation of MB provides a relatively simple system, allowing studying the morphogenesis of stable microtubule arrays.

The key question in MB biogenesis is whether the nucleation of MB microtubules arises from microtubule organizing centers (MTOCs) or from microtubule-nucleating activity located at the membrane skeleton or at plasma membrane. Although MTOCs in differenti-

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ating chicken erythroid cells are present, such structures are missing in mature erythrocytes. However, the MB of mature chicken erythrocytes can reform after cold depolymerization followed by rewarming, and it is indistinguishable from that of untreated cells (Miller and Solomon, 1984; Kim et al., 1987). MBs were found to contain the microtubule-associated proteins tau (Murphy and Wallis, 1985; Sanchez and Cohen, 1994), which possibly have microtubule stabilizing or bundling function, and syncolin that might be involved in lateral interactions between microtubules (Feick et al., 1991) or interaction of microtubule bundles with membranes (Sanchez and Cohen, 1994). The mechanism of MB formation in differentiating erythroid cells is not fully understood.

The γ-tubulin (Oakley and Oakley, 1989) is the almost ubiquitous component of MTOC and participates in nucleation of microtubules (Oakley et al., 1990; Joshi et al., 1992). In animal cells, the vast majority of γ-tubulin belongs to cytoplasmic complexes. Large γ-tubulin complexes (γ-tubulin-ring complex; γ-TuRC) were first identified in *Xenopus* eggs (Zheng et al., 1995). They were also found in *Drosophila* embryos (Moritz et al., 1995) and in mammalian cells (Meads and Schroer, 1995; Moudjou et al., 1996; Détraves et al., 1997). In addition to γTuRC, there also exist smaller complexes (γ-tubulin small complex; γTuSC) (Moritz et al., 1998) that compose two molecules of γ-tubulin and one molecule each of GCP2 and GCP3 (γ-tubulin complex proteins) (Murphy et al., 1998; Oegema et al., 1999), which are homologues of the *Saccharomyces cerevisiae* proteins Spc97p and SPc98p associated with spindle pole bodies (Knop and Schiebel, 1997). The γ-TuRCs are formed by small complexes and by other proteins. Protein composition differs in various cell types. In addition to nucleation from centrosomes, the γ-TuRCs are also involved in regulating the dynamics of the microtubule minus ends (Wiese and Zheng, 2000). Tubulin binding sites on γ-tubulin were identified, and it seems that γ-tubulin itself may, under some circumstances, bind to the side of assembled microtubules (Llanos et al., 1999). The role of γ-tubulin in nucleation of MB microtubules in differentiating and mature chicken erythrocytes is unknown.

Here, we report on changes in the distribution of γ-tubulin during chicken erythrocyte differentiation and characterize the soluble γ-tubulin forms. As a source of embryonic erythrocytes, we took advantage of embryonic peripheral blood, which is easily accessible and contains mainly erythroid cells.

RESULTS

Distribution of Gamma-Tubulin in Erythroid Cells and Mature Erythrocytes

The distribution of γ-tubulin in differentiating erythroid cells was studied by using monoclonal antibodies raised against conservative peptide sequences from human γ-tubulin. Antibodies TU-30, TU-31, and TU-32 are directed against a peptide from the C-terminal region of the molecule, the antibody GTU-88 is directed against a peptide from the N-terminal region. The specificity of antibodies was confirmed by immunoblotting on whole cell lysates of erythroid cells isolated from blood of 5-day-old embryos and on lysates of erythrocytes from 56-day-old chickens. An example of immunoblot with antibody TU-32 is shown in Figure 1. Protein with relative electrophoretic mobility corresponding to 48 kDa of γ-tubulin was detected in both samples. However, its amount in older chickens was lower (Fig. 1, lanes 3–4). The same results were obtained with antibodies raised against the C-terminal region of human γ-tubulin as well as with antibody against the N-terminal region of the protein. The observed decrease during erythrocyte development was characteristic for γ-tubulin because the amount of immunodetected α-tubulin was basically unchanged (Fig. 1, lanes 5–6) and the amount of vimentin increased (Fig. 1, lanes 7–8).

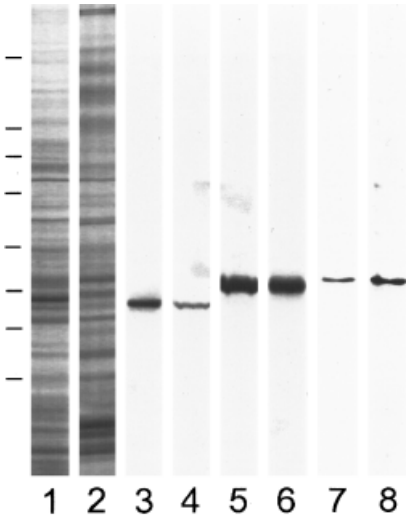

Fig. 1. Immunoblot analysis of differentiating erythrocytes. Whole cell extracts of erythroid cells from 5-day-old embryos (lanes 1, 3, 5, 7) and from erythrocytes of 56-day-old chickens (lanes 2, 4, 6, 8) were separated on 7.5–12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lanes 1–2, Coomassie Blue staining of separated proteins; lanes 3–8, immunostaining with antibodies TU-32 to γ-tubulin (lanes 3–4), TU-01 to α-tubulin (lanes 5–6), and VI-10 to vimentin (lanes 7–8). The same amounts of proteins were loaded (10 μg/lane). Bars on the left margin indicate positions of molecular mass markers (from the top to the bottom: 205 kDa, 116 kDa, 97.4 kDa, 84 kDa, 66 kDa, 55 kDa, 45 kDa, and 36 kDa).

To acquire detailed data on the course of γ-tubulin changes during erythrocyte differentiation, its level was determined at various stages of embryonic development. As γ-tubulin is necessary for the formation of mitotic spindle, the number of mitotic cells was estimated. Mitotic cells were most numerous on embryonic day (ED) 3 (4.33 ± 1.59%). The number of mitotic cells decreased to 0.43 ± 0.21% on ED 10, and almost no mitotic cells were detected on postnatal day 1 (0.03 ±

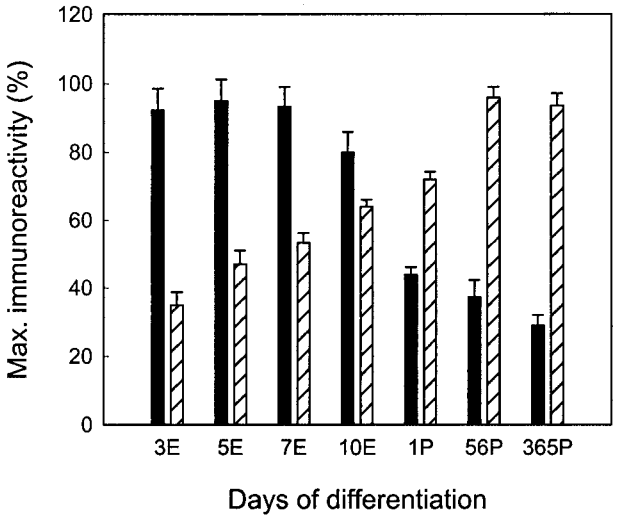

Fig. 2. Comparison of the expression of γ-tubulin and vimentin in differentiating erythrocytes as detected by densitometric measurements of immunoblots. The 3E, 5E, 7E, and 10E denote different days of embryonic development; 1P, 56P, and 365P denote different days of postnatal development. Each column represents the mean ± SE of duplicate readings of three blots. Black columns, γ-tubulin; shaded columns, vimentin.

0.03%). In the tested time period, the maximal immunoreactivity for γ-tubulin was attained between ED 3–7. Its level was reduced to approximately 45% of the maximum on postnatal day 1 and fell to approximately 30% of the maximum in erythrocytes isolated from adult animals. The level of vimentin increased during the observed period and reached its maximum on postnatal day 56. High level of vimentin was also detected on day 365. A comparison of γ-tubulin and vimentin changes in time is shown in Figure 2. These data show that γ-tubulin is still present in erythrocytes isolated from 1-year-old chickens.

Triple immunofluorescence with polyclonal antibody against αβ-tubulin dimer, monoclonal antibody against γ-tubulin, and DNA-binding dye revealed that γ-tubulin in erythroid cells of 5-day-old embryos was detectable not only in MTOCs in the perinuclear region but also on MB. Diffuse staining in the cytoplasm was also observed. In dividing cells, γ-tubulin was located on spindle poles of mitotic spindle, and also along the length of microtubules in the form of discrete dots. Interphase cells contained MTOCs that nucleated microtubules, which then formed MB under the plasma membrane (Fig. 3A–C). The MB in erythroid cells from 5-day-old embryos was thicker in comparison with later developmental stages. In 10-day-old embryos, the erythroid cells with thick MB, corresponding to primitive erythroid cells as to their shape and size, represented only a minority of erythrocyte population. Cells with MTOC nucleating microtubules were very rare. At that differentiating stage, cells started to appear that apparently did not have MTOC, because no γ-tubulin staining was detected in the perinuclear region of these cells (arrows in Fig. 3D–F). Due to the cell shape and thin MB, such cells represent most probably the final mature forms of the first wave of definitive erythroid cells. γ-Tubulin was also detected on MBs, but the intensity of staining varied among individual cells. An intensive staining of MB was found in cells with prominent labeling of MTOC, whereas the staining was weak in cells without MTOC. Surprisingly, the polyclonal antibody used against αβ-tubulin dimer did not decorate MTOCs that were brightly stained with anti–γ-tubulin antibody. In erythrocytes of 56-day-old chickens, γ-tubulin was not detectable by immunofluorescence in the perinuclear region or on MB (Fig. 3G–I). The same staining pattern of γ-tubulin in differentiating erythrocytes was observed with all monoclonal antibodies used.

To rule out the possibility that the location of γ-tubulin on MB reflects only an unspecific association of soluble cytoskeletal proteins with this prominent cellular structure, or possibly an unspecific sticking of primary and secondary antibodies, erythroid cells of 10-day-old embryos were stained with anti-vimentin antibody. Monoclonal antibody VI-10 (immunoglobulin M [IgM]) decorated only the filamentous network around the nuclei, and no staining of MB was detected (not shown). To learn more about the nature of γ-tubulin association with MB, cells were first extracted with 0.2% Triton X-100 in MSB containing 5 μM Taxol and the resulting cytoskeleton was incubated for 10 min in MSB containing 50, 100, 250, and 500 mM NaCl before fixation. γ-Tubulin staining of MB was observed after incubation of extracted cells in 250 mM NaCl. The majority of extracted cells incubated in 500 mM NaCl were detached from coverslips, but in the remaining cells γ-tubulin was still present on MB.

Redistribution of Gamma-Tubulin After Cold Depolymerization of MB

To investigate whether γ-tubulin is involved in nucleation of MB in erythroid cells from 10-day-old embryos, microtubules were depolymerized by cold treatment and the temperature was subsequently raised to 37°C to induce re-growth of microtubules. Samples were fixed at different time intervals. When cold-treated cells were directly extracted on ice by Triton X-100 and fixed, γ-tubulin was found in large clusters around the nucleus; diffuse staining was also observed. No staining of the cell periphery was detected. Similarly, staining with polyclonal antibody against tubulin dimers revealed tubulin clusters and diffuse staining, but the labeling was less intense than that seen with anti–γ-tubulin antibody (Fig. 4A–C). When cold-treated cells were directly extracted by Triton X-100 at 37°C and then fixed, microtubules started to form in some cells at the periphery. When cold-treated cells were incubated for 1 min at 37°C, different staining patterns were observed. There were cells without microtubules (~20%), cells with microtubules growing at

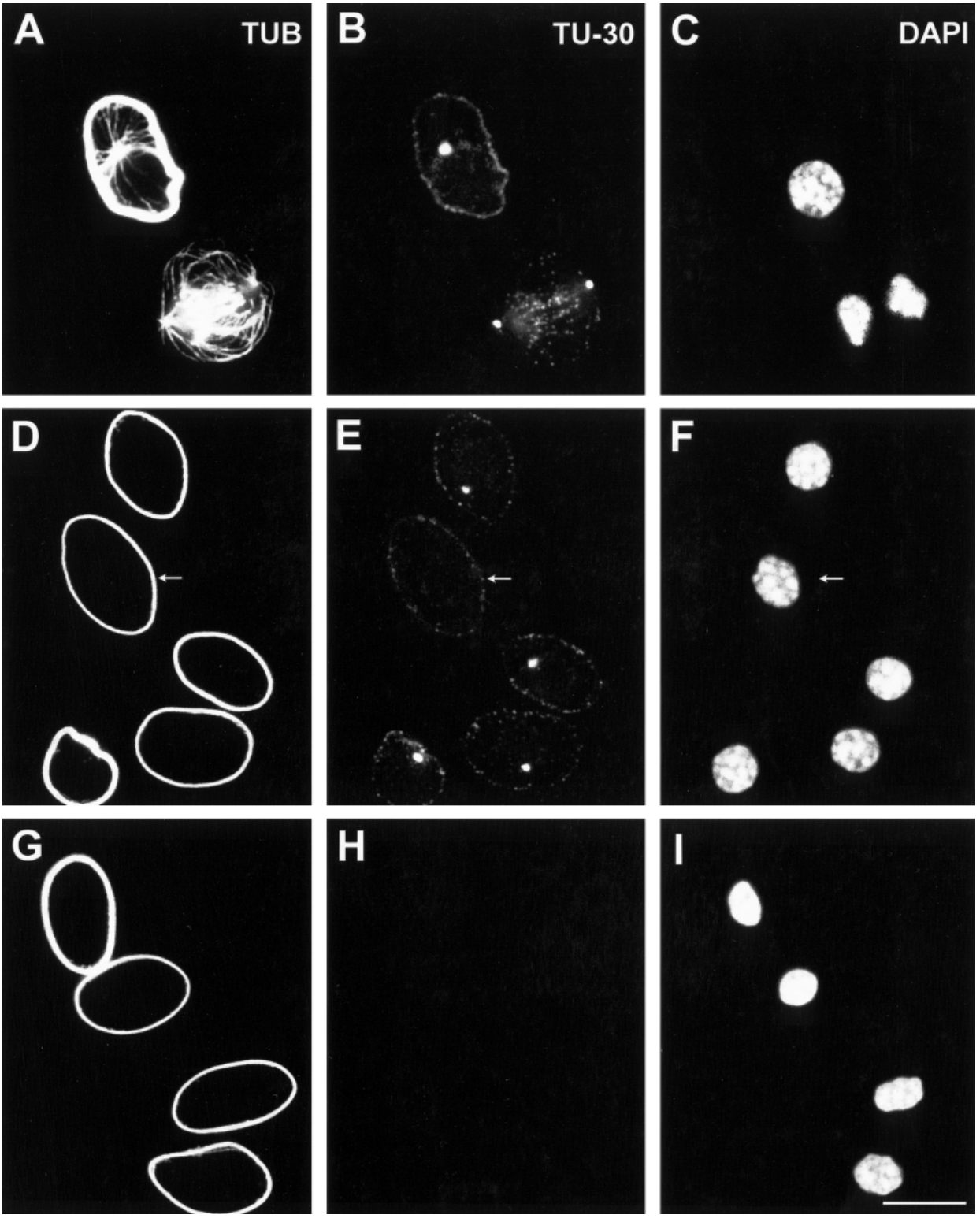

Fig. 3. Immunofluorescence triple-label staining of chicken erythrocytes in various stages of differentiation with anti–γ-tubulin antibody. Erythroid cells from 5-day-old embryos (**A–C**), 10-day-old embryos (**D–F**), and mature erythrocytes from 56-day-old chickens (**G–I**) were stained with polyclonal antibody TUB against αβ-tubulin dimers (A,D,G), monoclonal antibody TU-30 against γ-tubulin (B, E, H) and DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI; C,F,I). Cells were extracted with Triton X-100, fixed in formaldehyde and post-fixed in cold methanol. Arrows denote the same positions. Scale bar = 10 μm in I (applies to A–I).

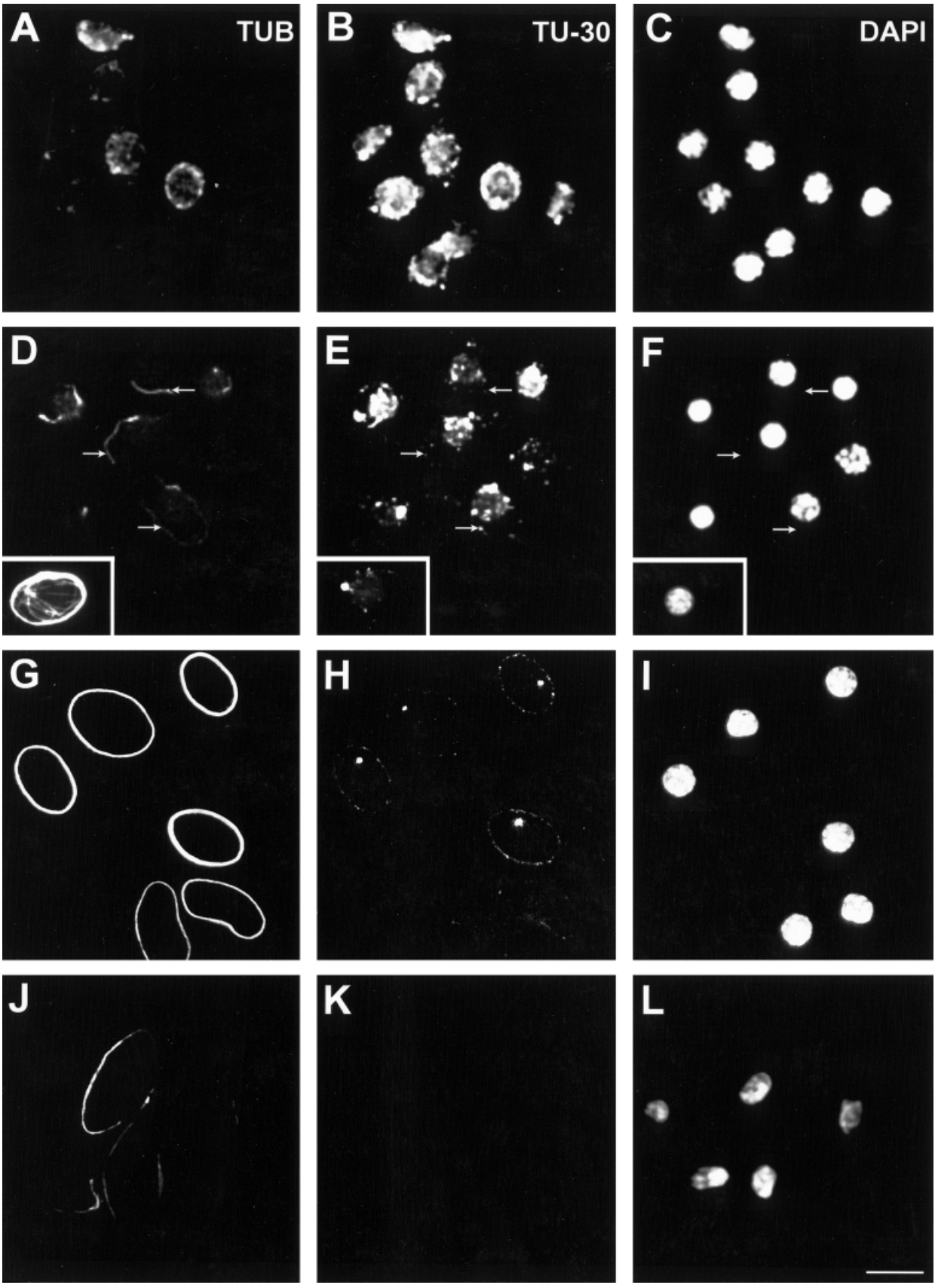

Fig. 4. The distribution of γ-tubulin in cells recovering from cold treatment. **A–I:** Erythroid cells from 10-day-old embryos. **J–L:** Erythrocytes from a 56-day-old chickens. Cells were incubated on ice for 16 hr and then fixed on ice (A–C) or after cold treatment incubated at 37°C for 1 min (D–F,J–L) or for 20 min (G–I) before fixation. Immunofluorescence triple-label staining with polyclonal antibody TUB against αβ-tubulin dimers (A,D,G,J), monoclonal antibody TU-30 against γ-tubulin (B,E,H,K), and DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI; C,F,I,L). Inserts in D–F document the re-growth of microtubules from microtubule organizing centers. Cells were extracted with Triton X-100, fixed in formaldehyde, and post-fixed in cold methanol. Arrows denote the same positions. Scale bar = 10 μm in L (applies to A–L).

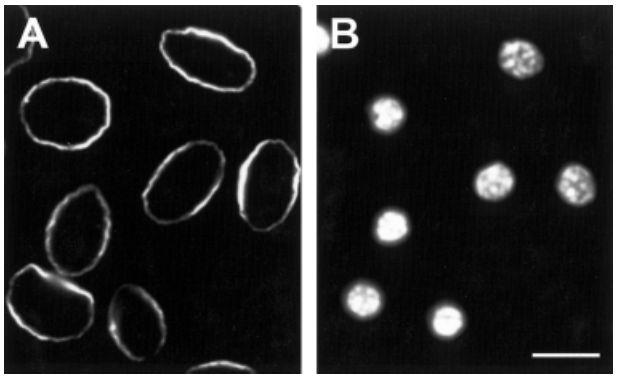

Fig. 5. Immunofluorescence staining of cold-treated erythroid cells from 10-day-old embryos. Cells were incubated on ice for 16 hr and then fixed on ice. Staining of actin with rhodamine-labeled phalloidin (**A**) and DNA-binding dye (**B**). Cells were extracted with Triton X-100, fixed in formaldehyde, and post-fixed in cold methanol. Scale bar = 10 μm in B (applies to A,B).

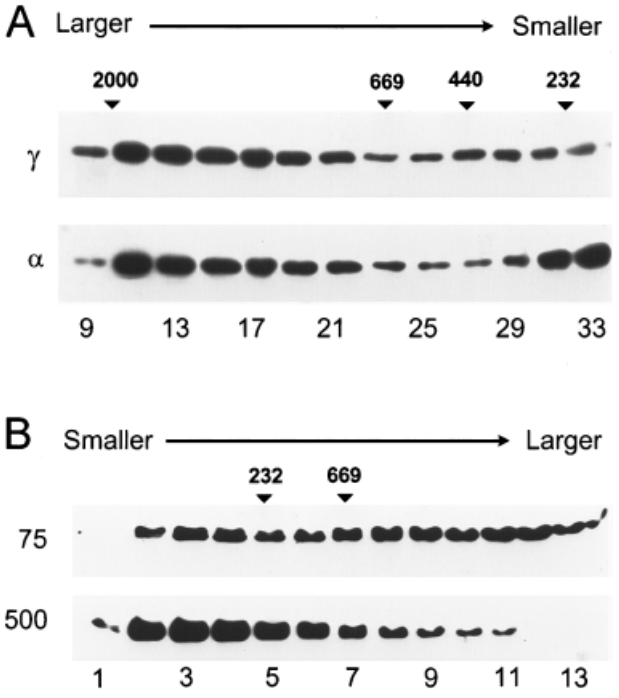

Fig. 6. Behavior of γ-tubulin during Superose-6 gel filtration (A) and sucrose density gradient centrifugation (B) of erythrocyte cell extracts from 3-day-old chickens. **A:** Collected fractions were concentrated, separated, and immunoblotted with antibody against γ-tubulin (γ) and α-tubulin (α). Numbers from 9 to 33 denote individual column fractions. **B:** Samples were sedimented through 5–40% sucrose gradient in the presence of 75 mM (75) or 500 mM (500) NaCl; fractions were separated and immunoblotted for γ-tubulin. Numbers from 1 to 13 denote individual fractions. Proteins were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Calibration standards in kilodaltons are indicated on the top.

the cell periphery (~ 55%), and cells with thin MB (~25%). However, no prominent staining of nucleation centers was detectable with anti–γ-tubulin antibody. On the other hand, faint γ-tubulin dots were visible on formed microtubules (arrows in Fig. 4D–F). Occasionally, it was possible to find cells that contained MTOC near the nucleus from which microtubules emanated. In such cells, thick MB had already been formed. γ-Tubulin was then located both on the MTOC and on the newly formed MB in the form of small spots (inserts in Fig. 4D–F). After 20 min, typical MBs, indistinguishable from those of untreated cells, were visible in the majority of cells (~95%). γ-Tubulin was located both in MTOCs, if present in examined cells, and as dots on MB. No clusters but diffuse staining was detected with anti–γ-tubulin antibody in the cytoplasm (Fig. 4G–I). The staining pattern of γ-tubulin in cold-treated cells was the same with all monoclonal antibodies used.

The formation of γ-tubulin clusters after depolymerization of microtubules was typical for embryonic erythroid cells. The same type of experiment performed with erythrocytes of 56-day-old chickens failed to detect clusters of γ-tubulin and γ-tubulin dots on newly formed microtubules after 1 min of re-growth at 37°C (Fig. 4J–L).

To rule out the possibility that tubulin aggregations reflects potential blebs on the surface of cold-treated cells, the cells were stained with rhodamine-labeled phalloidin that reacts with F-actin, which is not depolymerized by cold. F-actin was located on extracted cells on the cell periphery, and no substantial changes in morphology were observed (Fig. 5).

Gamma-Tubulin Complexes

Mature erythrocytes did not exhibit any cytoskeletal structures stained with anti–γ-tubulin antibodies, whereas blots from whole extracts showed the presence of γ-tubulin. Therefore, experiments were performed to determine whether γ-tubulin also exists in soluble cytoplasmic form. Size distribution of γ-tubulin in supernatants after Triton X-100 extraction of erythrocytes from 3-day-old chickens was assessed by gel filtration chromatography on Superose-6 column. Immunoblotting experiments with separated fractions revealed that γ-tubulin was distributed in a large zone and was present in complexes of various sizes. The majority of immunoreactivity was found in complexes with high molecular weight. Similar size fractionation was also found for α-tubulin; however, lower molecular weight complexes were found to prevail here (Fig. 6A). On the other hand, the heat shock protein Hsp70 was absent in high-molecular weight complexes, indicating that not all cytoplasmic proteins had the same size distribution under the used separation conditions (not shown). When Triton X-100 supernatants containing 75 mM NaCl were fractionated by sedimentation in 5–40% sucrose gradients, γ-tubulin was again present in complexes of various sizes. Large complexes, however, were disrupted in samples containing 500 mM NaCl (Fig. 6B). Combined data, thus, indicate that chicken γ-tubulin is present in high-molecular com-

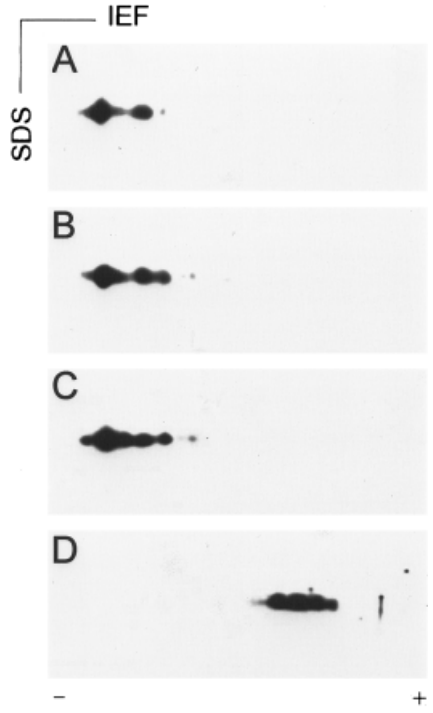

Fig. 7. Immunoblot analysis of erythrocyte cell extracts by two-dimensional electrophoresis. Immunostaining of erythroid cells from 5-day-old embryos (**A**), 10-day-old embryos (**B**), and mature erythrocytes from 56-day-old chickens (**C**,**D**) with anti–γ-tubulin antibody GTU-88 (A–C) and anti-α-tubulin antibody TU-01 (D). The basic and acidic ends in the first dimension are marked by minus and plus, respectively. The figures depict the same region in the first dimension. SDS, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

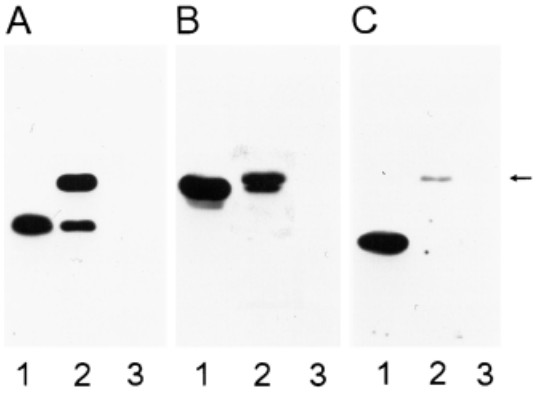

Fig. 8. Immunoprecipitation of erythrocyte cell extracts from 3-day-old chickens with anti–γ-tubulin antibody. Cell extracts were precipitated with anti–γ-tubulin antibody TU-31 (IgG2b) bound to immobilized protein A. Proteins remaining after precipitation (lane 1), immunoprecipitated proteins (lane 2), and proteins bound to protein A without antibody (lane 3) were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. **A:** Immunostaining with anti-γ-tubulin antibody GTU-88. **B:** Immunostaining with anti–α-tubulin antibody TU-01. **C:** Immunostaining with control anti-actin antibody. Arrow indicates the position of heavy chains of immunoglobulin G.

plexes and that their size is sensitive to salt concentration.

Two-dimensional analysis of Triton X-100 extracts revealed that soluble forms of γ-tubulin existed in multiple charge variants (Fig. 7A–C) that have their isoelectric points more basic in comparison with α-tubulin isoforms (Fig. 7D). The number of γ-tubulin charge variant increased in the course of differentiation, and at least six isoelectric variants of γ-tubulin were detected by immunoblotting in mature erythrocytes. In all tested differentiation stages, the isoelectric point of the major γ-tubulin isoform was 5.88.

Immunoprecipitation experiments showed that γ-tubulin could be specifically precipitated from Triton X-100 extracts of 3-day-old chicken erythrocytes by monoclonal antibody TU-31 (IgG2b) (Fig. 8A, lane 2). No binding of γ-tubulin to immobilized protein A was detected (Fig. 8A, lane 3), and the control antibody NF-09 (IgG2b) gave no precipitation of γ-tubulin. Probing of immunoprecipitated material with anti–α-tubulin antibody revealed the presence of α-tubulin in γ-tubulin complexes (Fig. 8B, lane 2). The band corresponding to α-tubulin was just under the band of immobilized TU-31 antibody (arrow in Fig. 8), so it could potentially reflect proteolytic fragmentation of the antibody. However, no band in this position was detected when the precipitated material was probed only with peroxidase-labeled anti-mouse antibody. When probed with anti–β-tubulin antibody TUB 2.1, specific staining was also detected (not shown). To rule out the possibility of an unspecific attachment of cytoplasmic proteins to the γ-tubulin complexes, immunoprecipitated material was probed with anti-actin antibody. In that case, no actin was detected (Fig. 8C, lane 2). When probed with anti-Hsp 70 antibody, no coprecipitation of this protein was found either (not shown).

Because the above-mentioned experiments indicated that tubulin dimers could be present in γ-tubulin complexes, immunoprecipitation was also performed with immobilized anti–α-tubulin antibody TU-16 (IgM). This antibody specifically precipitated α-tubulin (Fig. 9A, lane 2), and no binding of α-tubulin to immobilized protein L was detected (Fig. 9A, lane 3). Probing of the immunoprecipitated material with anti–γ-tubulin antibody revealed that the α-tubulin complexes also contained γ-tubulin (Fig. 9B, lane 2). Control antibody VI-10 (IgM) gave no precipitation of γ-tubulin. To rule out the possibility that cytoplasmic proteins are unspecifically attached to the α-tubulin complexes, immunoprecipitated material was probed with anti-vimentin antibody. In that case, no vimentin was detected (Fig. 9C, lane 2). Similarly, probing with anti-actin antibody showed no coprecipitation of the relevant protein (not shown). The same immunoprecipitation results were obtained with 3-day-old and 56-day-old chicken erythrocytes.

DISCUSSION

The distribution of γ-tubulin during the development and maturation of MB was investigated by using

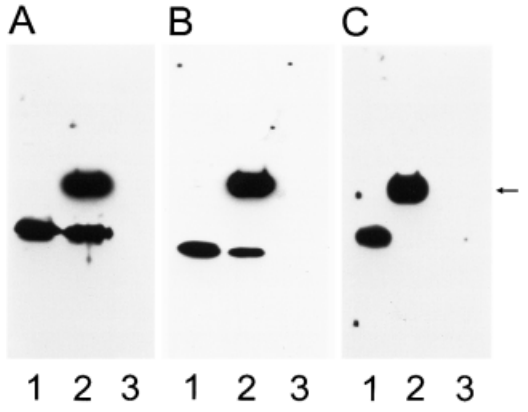

Fig. 9. Immunoprecipitation of erythrocyte cell extracts from 3-day-old chickens with anti–α-tubulin antibody. Cell extracts were precipitated with anti–α-tubulin antibody TU-16 (immunoglobulin M [IgM]) bound to immobilized protein L. Proteins remaining after precipitation (lane 1), immunoprecipitated proteins (lane 2), and proteins bound to protein L without antibody (lane 3) were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. **A:** Immunostaining with anti–α-tubulin antibody TU-01. **B:** Immunostaining with anti–γ-tubulin antibody GTU-88. **C:** Immunostaining with control anti-vimentin antibody VI-10. Arrow indicates the position of heavy chains of IgM.

chicken embryo erythropoiesis as a suitable model system. There are distinct developmental waves of erythroid cells distinguishable during chicken embryonic period. Cells of the primitive series appear 1.5–2 days after the start of fertilized egg incubation, and their first mature forms may be detected around ED 5. At that time, first immature cells of the definitive erythroid series appear in circulation and gradually replace the elements of primitive lineage. On ED 10, mainly definitive erythroid cells at different stages of differentiation are present in the blood and degenerating primitive erythrocytes constitute only approximately 10% of the blood cell population. Starting from ED 15 only cells of definitive series are exclusively present (Bruns and Ingram, 1973). Previous immunofluorescence studies indicated that no MTOC were detectable among chicken circulating erythroid cells on ED 5 and beyond (Kim et al., 1987). Our staining with anti–γ-tubulin antibodies clearly demonstrated the presence of MTOC in the majority of erythroid cells isolated from blood of 10-day-old embryos. However, by using a general polyclonal antibody against αβ-tubulin dimer, no such structures were detected. On the other hand, antibody against the detyrosinated form of α-tubulin, which is found in stable microtubule arrangements, was capable of revealing faint staining of MTOC at that developmental stage (E. Dráberová, unpublished results).

The location of γ-tubulin on stable microtubule arrangements of MB at early stages of development was an unexpected finding. Cold-depolymerization, which disrupted microtubules of MB, also abolished staining of the cell periphery with anti–γ-tubulin antibody, indicating that γ-tubulin is not associated with peripheral skeleton components. Differential localization of γ-tubulin on MB in the course of erythrocyte maturation suggests that the binding properties of γ-tubulin or it complexes must undergo substantial changes. Alternatively, the distinct staining can reflect changes in protein composition of MB proteins during maturation of MB (Murphy and Wallis, 1985; Feick et al., 1991; Zhu et al., 1995). γ-Tubulin has been found to be a microtubule minus-end binding molecule in not-anchored microtubules (Li and Joshi, 1995; Leguy et al., 2000). On the other hand, in vitro prepared γ-tubulin peptides were capable of binding along the length of assembled microtubules without interference with tubulin polymerization (Llanos et al., 1999). Although γ-tubulin in animal cells in vivo was found on microtubules of mitotic spindle as well as in midbodies (Julian et al., 1993; Lajoie-Mazenc et al., 1994; Nováková et al., 1996) and in plants even on all microtubule structures (Liu et al., 1993; Binarová et al., 1998), its localization on long microtubules in vertebrate cells was not reported. As the association of γ-tubulin with MB has only a transient character, γ-tubulin probably does not play any stabilizing role during MB maturation. It also does not reflect the binding to the minus ends of microtubules, as MB in erythrocytes are formed by a single or only a few extremely long microtubules (Cohen, 1991). Staining may, however, signify the storage form, inactive form, or both, of γ-tubulin.

The re-growth experiments on erythroid cells from 10-day-old embryos showed that there were no definite initiation sites at the cell periphery that contained γ-tubulin. Although we did not observe any clear γ-tubulin signal at the ends of growing microtubules, we cannot rule out that γ-tubulin is located on minus-ends of growing microtubules in erythroid cells or matured erythrocytes, but its detection is below the limit of fluorescence microscopy. Our data also support previous findings that there must be peripheral determinants of organization of MB microtubules that are independent of MTOC (Miller and Solomon, 1984; Kim et al., 1987). It is possible that proteins associated with microfilaments, which in erythrocytes colocalize with MB, could be directly or indirectly involved in such organization (Birgbauer and Solomon, 1989; Winckler et al., 1994). The results of re-growth experiments also strengthen the notion that γ-tubulins or their complexes from embryonic erythroid cells and from postnatal erythrocytes have different properties.

There were no cytoskeletal structures stained in immunofluorescence with anti–γ-tubulin antibodies in postnatal erythrocytes, and γ-tubulin was present only in soluble cytoplasmic pools. Soluble γ-tubulin occurred in complexes of various size and high molecular complexes dissociated in the presence of high salt concentration. Salt dissociation is a general property of γ–tubulin complexes in various species (Wiese and Zheng, 1999). Gel filtration also showed that αβ-tubulin dimers were eluted through elution profile of γ-tubulin, indicating heterogeneity in the size of complexes in

which αβ-tubulin dimers could be involved. A wide range of size distribution of tubulin dimers was observed in mammalian cell extracts (Moudjou et al., 1996). Size heterogeneity of erythrocyte γ-tubulin complexes could signify the presence of different complexes characterized by distinct polypeptides or self-association of γ-tubulin complexes (Détraves et al., 1997).

Precipitation experiments with Triton X-100 extracts showed that γ-tubulin indeed formed complexes with αβ-tubulin dimers. The GTU-88 monoclonal antibody directed against amino acid region γ38-53 failed to precipitate γ-tubulin. This finding suggests that this region is not accessible to the antibody binding in contrast to the region γ434-449 recognized by the TU-31 antibody. An association of soluble γ-tubulin with αβ-tubulin dimers was confirmed by precipitation with anti–α-tubulin antibody resulting in a coprecipitation of γ-tubulin. Variable amounts of αβ-tubulin dimers have been reported to coprecipitate with γ-tubulin in preparations from *Xenopus* embryos (Zheng et al., 1995), sheep brain (Détraves et al., 1997), and cells of lymphoblastic cell line KE37 (Moudjou et al., 1996). In other sources, no association of γ-tubulin with tubulin dimers was found (Wiese and Zheng, 1999). It is possible that the interaction of γ-tubulin with αβ-tubulin dimers in cells is weak and can be modified by sample preparations or by procedures used for the assessment of the composition of γ-tubulin complexes. At present, we do not know whether γ-tubulin in erythrocyte lysates interacts with other proteins beside tubulin dimers. Specific elution of proteins precipitated with TU-31 antibody (directly bound to carrier) with excess of the peptide, used for immunization, showed only traces of proteins in the position of tubulins and immunoglobulins when sensitive silver staining of proteins was applied (V. Sulimenko, unpublished results). This finding suggests that the association constant of TU-31 antibody for erythrocyte γ-tubulin is very high.

What the function of soluble γ-tubulin in matured erythrocytes actually is remains to be elucidated. However, the majority of αβ-tubulin dimers (~80%) also appear in adult erythrocytes in unassembled pools (Kim et al., 1987) and they easily form, due to specific properties of tubulin subunits, ring oligomers in vitro (Murphy and Wallis, 1985; Trinczek et al., 1993). It was suggested that tubulin oligomer pools in erythrocytes might be important for maintaining a low dimer concentration and, hence, nucleation rates to generate long microtubules (Murphy and Wallis, 1985). γ-Tubulin or its complexes could then stabilize tubulin oligomers.

The tubulin heterodimers are more homogeneous in avian erythrocytes than in other cell types (Joshi et al., 1987; Pratt and Cleveland, 1988), and posttranslational modifications are also less complex than in other cell types (Rüdiger and Weber, 1993). Surprisingly, multiple charge variants were detected with anti–γ-tubulin antibody, and the number of these isoforms increased during differentiation. Four γ-tubulin isoforms were recognized by two-dimensional electrophoresis in sheep brain (Détraves et al., 1997) and an elongated γ-tubulin signal with two major spots was detected in human lymphoblastic cell line (Moudjou et al., 1996). To date, only two γ-tubulin genes were identified in various species (Wise et al., 2000). These data suggest that γ-tubulin, like the α- and β-tubulin counterparts, could be subject to posttranslational modification(s). We also observed multiple α-tubulin isoforms that had not been previously detected in avian erythrocytes by two-dimensional electrophoresis (Murphy and Wallis, 1983) or by isoelectric focusing (Rüdiger and Weber, 1993). A higher number of α-tubulin isoforms could result from the use of immobilized pH gradient 4–7 in combination with sensitive immunostaining and chemiluminescent detection. An increased modification of γ-tubulin in the course of differentiation could result in the observed lower binding of γ-tubulin or its complexes to MB and in differential formation of γ-tubulin clusters in cold-treated cells.

In conclusion, the presented data indicate that the subcellular localization of γ-tubulin changes in the course of differentiation and that in postmitotic nucleated erythrocytes γ-tubulin persists in soluble form in complexes of various sizes. γ-Tubulin is not present in distinct nucleation structures at the cell periphery, and developmentally regulated posttranslational modification(s) of γ-tubulin could modify its binding properties.

EXPERIMENTAL PROCEDURES

Materials

Immobilized Protein A Plus, Immobilized Protein L Plus, and SuperSignal WestPico Chemiluminescent reagents were bought from Pierce (Rockford, IL). Protease inhibitors and molecular weight markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were from Sigma-Aldrich (Prague, Czech Republic). The gel filtration calibration kit contained Blue Dextran 2000, thyroglobulin ($M_r = 669 \times 10^3$), ferritin ($M_r = 440 \times 10^3$), catalase ($M_r = 232 \times 10^3$), and aldolase ($M_r = 158 \times 10^3$). Superose-6, IPG buffer (pH 4–7), and calibration kits for isoelectric focusing were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Antibodies

The following monoclonal antibodies were used: TU-01 (IgG1) against α-tubulin (Viklický et al., 1982); TU-16 (IgM) against α-tubulin (Dráberová and Dráber, 1998); TUB 2.1 (IgG1) against β-tubulin (Sigma Cat. No. T4026); TU-30 (IgG2b), TU-31 (IgG2b), TU-32 (IgG1) anti-peptide antibodies prepared against EYHAATRPDYISWGTQ peptide corresponding to the human γ-tubulin sequence 434-449 (Nováková et al., 1996); GTU-88 (IgG1) anti-peptide antibody prepared against EEFATEGTDRKDVFFY peptide corresponding to the human γ-tubulin sequence 38-53 (Sigma Cat. No. T6557); VI-10 (IgM) against vimentin (Dráberová et al., 1999b); N27F3-4 (IgG1) against heat shock pro-

tein Hsp 70 (StressGen, Cat. No. SPA-820); and NF-09 (IgG2a) against neurofilament protein (Dráberová et al., 1999a). Actin was detected by affinity-purified rabbit anti-actin antibody (Sigma Cat. No. A2066) and microtubules were detected by affinity-purified rabbit anti-tubulin antibody (Dráber et al., 1991). Anti-mouse Ig antibodies conjugated with horseradish peroxidase were purchased from Promega Biotec (Madison, WI), lissamine rhodamine-conjugated anti-mouse Ig antibody and fluorescein isothiocyanate-conjugated anti-rabbit Ig antibody was from Jackson Immunoresearch Laboratories (West Grove, PA).

Cells and Preparation of Cell Extracts

Outbred Grey Leghorn chickens (*Gallus domesticus*) were used as a source of chicken blood. During the embryonic period, blood samples were obtained from the dorsal aorta (3- and 5-day-old embryos) and from arteria vitellina (7- and 10-day-old embryos). Heart blood was withdrawn from chicks on day 1, 56, and 365 after hatching. Blood samples were collected into 0.1% BSA in 110 mM NaCl, pH 8.0 (3- and 5-day-old embryos) or into 0.1% BSA in 145 mM NaCl, pH 8.0 (7- and 10-day-old embryos). Citrate-saline solution (final concentration 145 mM NaCl, 34 mM tri-sodium citrate, pH 7.6) was used for collection of blood from hatched chicks. Conventional blood smears were prepared for histochemical evaluation of mitotic cells. The percentage of mitotic cells (mean ± SD) was calculated from four independent samples (3,000 cells/sample).

For preparation of cell extracts, blood samples were spun down at 220 × *g* for 5 min at room temperature, the supernatant and buffy coat were removed by aspiration, cells were resuspended in phosphate-buffered saline (PBS), pelleted again at room temperature, and washed in PBS three times. Washed cells contained more than 99% of erythrocytes as confirmed by conventional hematology examination.

Whole cell extracts for SDS-PAGE and for immunoprecipitation were prepared as follows: pelleted washed cells were resuspended in one volume of cold MEM buffer (0.1 M Mes adjusted to pH 6.9 with KOH, 2 mM EGTA, 2 mM $MgCl_2$) supplemented with protease (1 μg/ml each of leupeptin, aprotinin, antipain, pepstatin and 1 mM AEBSF) and phosphatase inhibitors (1 mM each of NaF and Na_3VO_4) and with Triton X-100 to final concentration of 0.4%. Cells were extracted 10 min at 4°C, suspension was then spun down (21,000 g, 15 min, 4°C), and supernatant was collected. The remaining pellet was resuspended in cold 0.4% Triton X-100 in MEM buffer supplemented with protease and phosphatase inhibitors and disrupted on ice by sonication for 5 × 10 sec (amplitude 30) with 500-watt Ultrasonic homogenizer (Cole-Parmer, Vernon Hills, IL) equipped with a microtip probe. The suspension was spun down (21,000 × *g*, 15 min, 4°C), supernatant collected and pooled with the supernatant after extraction.

For gel filtration chromatography, pelleted washed cells were resuspended at 4°C in 1 volume of precooled Hepes buffer (50 mM Na-Hepes, pH 7.6, 75 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, and 1 mM 2-mercaptoethanol) supplemented with 2% glycerol and protease and phosphatase inhibitors. Triton X-100 was added to the final concentration of 0.4%, and cells were permeabilized for 10 min at 4°C. Suspensions were spun down (21,000 × *g*, 15 min, 4°C), and supernatants were used for gel filtration.

For sucrose density gradient centrifugation, pelleted washed cells were resuspended at 4°C in 1 volume of precooled Hepes buffer supplemented with protease and phosphatase inhibitors, and with or without 500 mM NaCl. Triton X-100 was added to the final concentration of 0.4%, and cells were permeabilized for 10 min at 4°C. Suspensions were spun down (21,000 × *g*, 15 min, 4°C), and supernatants were used for gradient centrifugation.

Gel Filtration Chromatography

Gel filtration was carried out at 4°C on a 16.6 × 1.6 cm Superose-6 column (Amersham Pharmacia Biotech, Vienna, Austria) equilibrated in Hepes buffer supplemented with 2% glycerol (column buffer). Column buffer as well as the 0.25-ml samples was passed through a 0.22-μm filter. After loading, the column was eluted at 6 ml/hr and 0.5-ml aliquots were collected. Proteins were precipitated with chloroform-methanol (Wessel and Flugge, 1984), and air-dried pellets were then dissolved in 50 μl of SDS-PAGE buffer.

Sucrose Gradient Sedimentation

Sucrose gradient centrifugation was carried out as described (Moritz et al., 1998). The 5–40% sucrose gradients were poured as step density gradients (950-μl steps: 40, 30, 20, 10, and 5% sucrose) and allowed to diffuse into continuous gradients overnight at 4°C before use. The gradients were prepared in Hepes buffer (75 mM NaCl) or in Hepes buffer supplemented with NaCl to final concentration 500 mM. A 75-μl sample aliquot was loaded onto each gradient, and the gradients were centrifuged at 50,000 rpm in a Beckman SW55 rotor for 4 hr at 4°C. Gradients were fractionated from the top into 14 fractions. Protein standards (0.5 mg/ml each) were loaded in an equivalent volume and were run in parallel over identical gradients for each experiment. Samples for SDS-PAGE were prepared by mixing with 5× concentrated SDS-PAGE sample buffer.

Immunoprecipitation

Immunoprecipitation was performed as previously described (Dráberová and Dráber, 1993). Cell extracts were incubated with beads of (1) immobilized protein A saturated with anti–γ-tubulin antibody TU-31, (2) immobilized protein A saturated with control antibody NF-09, (3) immobilized protein A alone, (4) immobilized protein L saturated with anti–α-tubulin antibody

TU-16, (5) immobilized protein L saturated with control anti-vimentin antibody VI-10, or (6) immobilized protein L alone. The antibodies were used in the form of culture supernatants to avoid immobilization of other mouse antibodies. 50 μl of sedimented beads were incubated under rocking at 4°C for 2 hr with 1.2 ml of the corresponding antibody, prepared by mixing 0.4 ml of concentrated supernatant with 0.8 ml of TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). The beads were pelleted by centrifugation at 5,000 × *g* for 1 min, washed four times (5 min each) in cold TBST, and further incubated under rocking for 3 hr at 4°C with 0.5 ml of cell extract diluted 1:1 with TBST. Thereafter, the beads were pelleted and washed four times (5 min each) in cold TBST before boiling for 5 min in 100 μl of SDS-sample buffer to release the bound proteins.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed according to Laemmli (1970). When whole cell extracts were analyzed, boiled samples were centrifuged at 22,000 × *g* for 10 min and separated on 7.5–12.5% gels; Coomassie Blue stained gels were densitometrically scanned. The amount of proteins in samples for subsequent immunoblotting experiments was adjusted, by using BSA as a standard, to 10 μg of proteins per blotted area in each lane. Proteins were electrophoretically transferred from gels onto nitrocellulose sheets and immunostained. Details of the immunostaining procedure are described elsewhere (Dráber et al., 1988). The antibodies TU-01, TU-30, TU-31, TU-32, and VI-10 in the form of spent culture supernatants were used undiluted, the antibody GTU-88, in the form of ascitic fluid, was diluted 1:4,000. The antibody against β-tubulin and antibody against Hsp70 were diluted 1:2,000, and antibody against actin was diluted 1:200. Bound antibodies were detected by incubation of blots with anti-mouse antibody conjugated with horseradish peroxidase diluted 1:10,000, and after washing with chemiluminescent reagents according to the manufacturer's directions. Autoradiography films X-Omat AR (Eastman Kodak, Rochester, NY) were quantified by densitometry using gel documentation system GDS 7500 and GelBase/GelBlot Pro analysis software (UVP, Upland, CA).

In two-dimensional electrophoresis, samples were first separated by isoelectric focusing on rehydrated 11-cm-long Immobiline DryStrip gels, pH gradient 4–7 (Amersham Pharmacia Biotech, Uppsala, Sweden) at 22,000 V hours on Multiphor II apparatus (LKB, Bromma, Sweden). The samples, Triton X-100 extracts in MEM buffer, were diluted 40 times in running solution composed of 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 20 mM DTT, and 2% IPG buffer, pH 4–7. Strips were immersed into protein-containing running solution, covered with mineral oil and allowed to equilibrate according to the manufacturer's instruction. The second dimension was performed on 8% SDS-PAGE and separated proteins were electrophoretically transferred onto nitrocellulose.

Immunofluorescence

Blood cells from chicken embryos and hatched chicks were washed three times in, respectively, corresponding collecting buffer and PBS. Washed cells were resuspended 1:1 in the corresponding buffer and thereafter diluted with the buffer 1:2,000 before loading to poly-L-lysine coated coverslips for 10 min at 37°C. In some cases, the attached cells were incubated on ice for 16 hr and then fixed or incubated at 37°C for various time intervals, ranging from 30 sec to 20 min, before cytoskeleton preparation. Extraction and fixation steps were carried out in MEM buffer supplemented with 4% PEG 6,000 (microtubule stabilizing buffer; MSB). Cells on coverslips were extracted for 1 min at 37°C with 0.2% Triton X-100 in MSB and the resulting cytoskeletons were fixed for 20 min at 37°C in 3% formaldehyde in MSB, followed by 10 min fixation in methanol at −20°C. Taxol-stabilized cytoskeleton was prepared by extracting the cells for 4 min at 37°C with 0.2% Triton X-100 in MSB containing 5 μM Taxol. Such preparations were then incubated for 10 min at 37°C with MSB containing 50, 100, 250, and 500 mM NaCl before formaldehyde and methanol fixation. Monoclonal antibodies were used as undiluted supernatants. For double-label immunofluorescence, the coverslips were incubated simultaneously for 45 min with affinity-purified rabbit antibody against the αβ-tubulin heterodimer (dilution 1:10) and with monoclonal antibodies against γ-tubulin. After washing, the slides were incubated simultaneously with fluorescein isothiocyanate–conjugated anti-rabbit Ig and lissamine rhodamine-conjugated anti-mouse Ig antibody, both diluted 1:100. Actin was detected with rhodamine-conjugated phalloidin. The preparations were mounted in Mowiol 4-88 supplemented with 1 μg/ml of 4,6-diamidino-2-phenylindole (DAPI) and n-propylgalate, and examined with an Olympus A70 Provis microscope equipped with 100× oil-immersion objective. Images were recorded with a Life Science Resources KAF 1400 cooled CCD camera. The control antibody NF-09 did not provide staining.

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