Abstract. Chicken erythrocyte beta tubulin, a tubulin variant with unique biochemical and assembly properties, is found to be specifically contained in two chicken blood cell types—erythrocytes and thrombocytes. The beta tubulin variant is absent or present in low amounts in a variety of white blood cell types and other body tissues, as determined by immunofluorescence microscopy and a semi-quantitative immunoblotting procedure. During differentiation in the marrow the beta tubulin variant appears suddenly in mid-stage erythrocytes of the blood, and that it first appears in developing erythroblasts at a time corresponding to the initiation of hemoglobin synthesis and marginal band formation. As observed by immunofluorescence microscopy, marginal band assembly appears to involve several distinct events—microtubule initiation and growth, consolidation into a band, and association with the cell membrane.

Chicken erythrocytes contain a beta tubulin variant that is distinct from the beta tubulin found in chicken brain (Murphy and Wallis, 1983a, b; Murphy et al., 1984). Our investigations on erythrocyte tubulin support the idea that different tubulin variants may have different functions, a concept first described by Stephens (1975) and later termed the multityubulin hypothesis by Fulton and Simpson (1976). In previous reports we demonstrated that purified erythrocyte tubulin exhibits different rate constants for subunit addition and loss (Rothwell et al., 1985a, b), has different values of critical concentration (Murphy and Wallis, 1983b; Rothwell et al., 1985a, b), is more alkaline and hydrophobic, and has fewer reactive sulfhydryls than does beta tubulin from chicken brain tissue (Murphy and Wallis, 1983a; Murphy et al., 1984; Luduena et al., 1985). Furthermore, erythrocyte beta tubulin appears to be the product of a unique beta tubulin gene as judged by peptide mapping and examination of overall amino acid composition (Murphy and Wallis, 1983a; Murphy et al., 1984) and by the recent observation of unique amino acid sequences in certain peptides (unpublished observations). The question therefore arises whether the chemical and physical properties of erythrocyte beta tubulin are related to its assembly and function in the cell and whether this tubulin variant is expressed in specific cell types.

In this paper we use a rabbit antibody to erythrocyte beta tubulin to determine if the tubulin variant we had previously isolated and characterized was specifically expressed in erythrocytes and to examine the relationship between beta tubulin expression and the process of marginal band formation in developing chicken erythroblasts. We found that the beta tubulin variant is exclusively expressed in erythrocytes and thrombocytes of the blood, and that it first appears in developing erythroblasts at a time corresponding to the initiation of hemoglobin synthesis and marginal band formation. As observed by immunofluorescence microscopy, marginal band assembly appears to involve several distinct events—microtubule initiation and growth, consolidation into a band, and association with the cell membrane.

Materials and Methods

Tubulin Antibodies

Rabbit antibodies that recognize both alpha and beta tubulin subunits (general tubulin antibody) were obtained using tubulin vinblastine crystals from sea urchin eggs as antigen as described previously by Fujiiwara and Pollard (1978). Tubulin antibodies were affinity purified as described below except that the tubulin affinity column contained phosphocellulose-purified chicken brain tubulin (Murphy and Wallis, 1983b; Weingarten et al., 1975). Antibodies specific for just the beta subunit of chicken erythrocyte tubulin were also obtained. Phosphocellulose-purified erythrocyte tubulin (Murphy and Wallis, 1983b; Weingarten et al., 1975) was reduced and alkylated as described by Crestfield et al. (1963) and chromatographed on hydroxyapatite (Bio-Rad Laboratories, Richmond, CA) in the presence of SDS as described by Lu and Eizenga (1978) and Little (1979). Purified beta tubulin subunits were mixed with Freund's adjuvant and injected subcutaneously into three rabbits (420 µg each for both primary inoculations and secondary boosts). The immune sera of all three rabbits reacted specifically with the erythrocyte beta tubulin subunit as determined by immunoblotting. The antigenicity of this subunit and its ability to elicit specific antibodies may be due to significant differences in its primary structure compared with other chicken beta tubulins (Murphy and Wallis, 1983a; Rothwell et al., 1985a).

Tubulin antibodies were affinity-purified on Sepharose columns containing phosphocellulose-purified erythrocyte tubulin. Tubulin antibodies were eluted from the column with 1 M acetic acid, neutralized to pH 4 with 1 M Tris, dialyzed against phosphate-buffered saline (PBS), and frozen until use.

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The Journal of Cell Biology, Volume 102, February 1986 628-635
Determination of Percent Tubulin Content in Chicken Tissues

The percent erythrocyte beta tubulin contained in various chicken tissues was determined by a semiquantitative immunoblotting procedure. Freshly dissected tissues of a 7-d-old chick were homogenized, centrifuged to prepare extracts, and mixed with boiling SDS gel sample buffer. SDS polyacrylamide gel electrophoresis (Laemmli, 1970) was performed at pH 9.1 to assure complete separation of the alpha and beta subunits of all tubulin species. After electrophoretic transfer onto nitrocellulose (Towbin et al., 1979), incubation in tubulin antibody and 125I-protein A, and autoradiography, the amounts of both total tubulin and erythrocyte-specific tubulin were determined by comparing the intensities of antibody labeling of tubulin bands in the tissue extracts with the amount of label on a concentration series of purified tubulin standards. By visual inspection of autoradiograms we determined that the amount of antibody binding was proportional to the amount of tubulin standard loaded on the gel (linear range, 1.5–150 ng). The amount of reactivity of the general tubulin antibody at the position of alpha tubulin was used to estimate total tubulin, and the amount of reactivity of specific beta tubulin antibody at the beta tubulin position was used to estimate the amounts of erythrocyte beta tubulin. The amount of total and specific tubulin in erythrocytes calculated by this method (0.5% of the total cellular protein) agrees well with our previously published estimates based on radioimmunoassay (0.6%), pelleting of taxol-stabilized microtubules (0.6%), and SDS gel electrophoresis (1.1%) (Murphy and Wallis, 1983b). We estimated the error of this method to be 10–20%.

Cell Staining and Immunofluorescence Microscopy

Blood from 1-wk-old chicks (0.2 ml) was collected into 10 ml citrate-saline (0.9% NaCl and 0.3% sodium citrate, pH 7.2). Bone marrow dissected from the long bones was minced in PBS containing 5 mM MgCl2 and dispersed by gentle pipetting, and cells were allowed to settle from the suspension and attach to the surfaces of polylysine-coated coverslips. Cells were rinsed, stabilized, and extracted with 4% polyethylene glycol and 0.5% Triton as described by Osborn and Weber (1982), fixed and dehydrated in 100% methanol, rehydrated with PBS, and labeled with affinity-purified antibodies to tubulin and fluorescent-labeled goat anti-rabbit antibody. Specimens were examined on a Leitz photomicroscope (E. Leitz, Inc., Rockleigh, NJ) equipped with a Zeiss oil immersion lens (phase contrast 63×, 1.4 numerical aperture Carl Zeiss, Inc., Thornwood, NY). To increase the proportions of erythroblasts at early stages, we induced anemia with phenylhydrazine (15 mg/kg, intramuscular injection), given on each of two consecutive days with bleeding on the third day.

Results

Tissue Specificity of Beta Tubulin Expression

Tubulin was quantitated in a variety of chicken tissues by a semiquantitative immunoblotting procedure using two tubulin antibodies. A rabbit antibody to sea urchin tubulin, which recognizes both alpha and beta tubulin subunits (general tubulin antibody), was used to estimate total tubulin (Fig. 1); a second rabbit antibody specific for erythrocyte beta tubulin was used to determine the amount of erythrocyte beta tubulin (Fig. 1). The results of the tissue survey are shown in Table I. Most chicken tissues were observed to contain only small amounts of erythrocyte beta tubulin, typically 0.5–1.0% of the total tubulin in the tissue. In organs containing large amounts of blood (liver, kidney, spleen) and/or considered to be hemopoietic organs (liver, pancreas, spleen), the amounts of erythrocyte beta tubulin were higher, ranging from 8 to 23%. In all the tissues examined, the amount of erythrocyte beta tubulin observed was in the range of that expected for erythrocyte contamination in the tissue. From these results we estimate that the amount of erythrocyte beta tubulin specifically expressed in nonerythroid tissues is low or absent.

Erythrocyte Beta Tubulin Is Specifically Expressed in Erythrocytes and Thrombocytes in the Blood and Bone Marrow

Immunofluorescence staining of chicken blood and bone marrow cells using general tubulin antibody labeled the marginal bands in erythrocytes and thrombocytes and centrosomal microtubules in other white blood cells (Fig. 2, A and B).

Table I. Tissue Survey for Erythrocyte Beta Tubulin

| Tissue/Organ | Total tubulin in extract | Erythrocyte beta tubulin in extract | Erythrocyte beta tubulin in total tubulin | Normalized |
|--------------|--------------------------|------------------------------------|------------------------------------------|------------|
| %            | %                        | %                                  | %                                       | %          |
| Adrenal glands | 1.9                      | .03                                | 1.6                                     | 1.5        |
| Erythrocytes  | 0.5                      | .03                                | 108.0                                   | 100.0      |
| Brain         | 13.3                     | .05                                | 0.4                                     | 0.4        |
| Bursa         | 3.8                      | .02                                | 0.5                                     | 0.5        |
| Kidney        | 0.3                      | .03                                | 10.0                                    | 9.2        |
| Liver         | 0.08                     | .01                                | 8.7                                     | 8.0        |
| Pancreas      | 0.08                     | .01                                | 12.5                                    | 11.6       |
| Testis        | 2.8                      | .05                                | 1.8                                     | 1.7        |
| Thymus        | 1.8                      | .02                                | 1.1                                     | 1.0        |
| Spleen        | 0.08                     | .02                                | 25.0                                    | 23.1       |

The percent tubulin content was obtained by comparing immunoblots of tissue extracts with known amounts of erythrocyte tubulin using rabbit antisera to sea urchin tubulin to determine the amount of tubulin and a rabbit antibody to erythrocyte beta tubulin to determine the amounts of erythrocyte beta tubulin. The percent erythrocyte beta tubulin was normalized with respect to erythrocytes, which were designated 100%.
Figure 2. Evidence for the specific localization of erythrocyte beta tubulin in erythrocytes and thrombocytes. Chicken bone marrow cells were labeled with general tubulin and erythrocyte beta tubulin antibodies. General tubulin antibody (A and B) labels centrosomal microtubules in granulocytes (G) and the marginal bands in erythrocytes and thrombocytes, which are not distinguishable in this preparation (not labeled). Erythrocyte beta tubulin antibody (C and D) labels only late maturing erythroblasts but not granulocytes (G) or early stage erythroblasts (E). Centrosomal microtubules containing the erythrocyte tubulin variant are indicated by an arrow in an erythroblast shown in D. Bar, 5 μm.
However, only erythrocytes (Fig. 2, C and D) and nucleated thrombocytes (Fig. 3) (cells functionally analogous to mammalian blood platelets) contained the specific tubulin variant that was primarily localized in the circumferential bundle of microtubules known as the marginal band. To identify thrombocytes unambiguously, it was necessary to prepare blood smears in the absence of anticoagulant agents. Aggregates of thrombocytes, which formed under these conditions, could be readily identified and were observed to be intensely labeled with the erythrocyte beta tubulin antibody as determined by immunofluorescence microscopy (Fig. 3). Chicken thrombocytes contain marginal bands and are known to be developmentally related to erythrocytes (Lucas and Jamroz, 1961). Microtubules in granulocytes (heterophils, eosinophils, basophils) were not stained by the beta tubulin antibody (Fig. 2, C and D). No beta tubulin variant was detected in cultured chicken lymphoblasts as determined by immunoblotting or in immature lymphocytes as determined by immunofluorescence microscopy. Other nonerythroid cells, identified by characteristic microscopic features, were also unlabeled. These included macrophages (autofluorescent phagosomes), osteoclasts (multinucleate cells), adipocytes (lipid droplets), and fibroblasts (elongate nuclei) (not shown). Thus, of the cells in the peripheral blood, expression of the beta tubulin variant was specific for erythrocytes and thrombocytes, both of which are developmentally related (Lucas and Jamroz, 1961) and contain marginal bands.

Patterns of immunofluorescence staining in bone marrow smears were also examined to determine if the beta tubulin variant was contained in early erythrocyte precursors and when in development the expression occurred. In chicken, erythrocyte precursors comprise 75–80% of the total marrow cell population (Lucas and Jamroz, 1961). Erythroblasts were readily identified by light microscopy after Jenner-Giemsa staining or by phase contrast microscopy in the absence of staining as large cells with large round nuclei and prominent nucleoli. The percent of red cell precursors could be increased to ~90% and the proportion of early stage erythroblasts brought from ~2–20% by inducing anemia with phenylhydrazine.

When bone marrow smears were stained with the general tubulin antibody, developing marginal bands in late-stage erythroblasts and thrombocytes (not distinguishable from each other in extracted preparations by phase contrast) and centrosomal microtubules in early blast cell stages were identified (not shown). Staining with the beta tubulin specific antibody was very different (Fig. 4). None of the early erythroblast stages (proerythroblasts, early erythroblasts) were intensely stained. Only later stages (mid and late stage erythroblasts, poly- and orthochromatophilic erythroblasts) contained labeled microtubules. In erythrocyte precursors antibodies were also distributed diffusely throughout the cytoplasm, on centrosomal microtubules, and on mitotic spindles of dividing erythroblasts at the terminal mitosis before marginal band formation (Fig. 4, indicated by stars). This observation indicates that the erythrocyte tubulin variant can perform several different functions in addition to its primary function which is to form the marginal band. Fluorescence was greatest in later erythroblast stages at the time of consolidation of microtubules into a marginal band. The fact that early erythroblasts do contain microtubules but contain little if any beta tubulin variant and the fact that at least 90–95% of the tubulin

Figure 3. Chicken thrombocytes contain erythrocyte beta tubulin. Undiluted fresh chicken blood was momentarily deposited on a coverslip before processing for immunofluorescence microscopy. Initial clotting and thrombocyte activation allowed definitive identification of small clumps of thrombocytes by phase contrast microscopy. Thrombocytes appear more intensely labeled than erythrocytes because of incomplete extraction of the erythrocytes in samples prepared by this method. Bar, 10 μm.

The Sequence of Events in Marginal Band Formation

Dispersed bone marrow cells from anemic chicks containing a high percentage of early erythroblast stages were allowed to attach to coverslips and were processed for immunofluorescence microscopy using the general tubulin antibody (Fig. 2, A and B) and the erythrocyte tubulin antibody (Fig. 2, C and D) to observe the sequence of morphological events in the formation of the marginal band. A sequence of stages as seen with the general tubulin antibody (in order to observe microtubules in all stages of development) is shown in Fig. 5. Large early erythroblasts contained few but otherwise normal looking centrosomal microtubules. At later developmental stages, beginning with the time of appearance of the beta tubulin variant, the following events were observed: (a) The cell size
Figure 4. Expression of the beta tubulin variant in developing marrow erythroblasts. Chicken bone marrow from an animal made anemic with phenylhydrazine was removed and smeared on a coverslip which was then processed to stabilize microtubules, labeled with beta tubulin antibody, and processed for immunofluorescence microscopy. Only mature erythrocytes and late stage erythroblast precursors are labeled with the antibody. The diffuse pattern of labeling is due to incomplete extraction of soluble tubulin monomer in these preparations. Early erythroblasts (large cells with large round nuclei and prominent nucleoli that are indicated with arrows), granulocytes (G), lymphocytes (L), and their precursors are not specifically labeled. Asterisks indicate dividing erythroblasts whose mitotic spindles also contain the beta tubulin variant. Bar, 5 μm.
became smaller, while the nucleus became both smaller and more heterochromatic. (b) Microtubules associated with the centrosome extended outwards forming a loose wreath that occupied the space between the nuclear membrane and the cell surface. The density of microtubules in the cytoplasm increased greatly during differentiation as the number of microtubules increased and the cell size decreased. These observations suggest that the nucleating activity of the centrosome may be important in the initial stages of marginal band formation. The presence of the erythrocyte tubulin variant in centrosomal microtubules at early stages of marginal band formation was determined by immunofluorescence staining of erythroblasts using the erythrocyte beta tubulin antibody (Fig. 2D) and by double immunofluorescence staining with both the general and specific tubulin antibodies (not shown). (c) Associations between microtubule ends and the centrosome became indistinct and eventually became lost or obscured; the microtubules became consolidated into an organized bundle. (d) Microtubules became associated into a highly compact bundle that was closely opposed to the cell membrane.

Discussion

Tissue Specificity of Tubulin Expression

Nucleated chicken erythrocytes contain a beta tubulin variant that is unique not only in its biochemical characteristics and properties of assembly but also in the specificity of its expression in the erythrocytes and thrombocytes of the blood. Examination of individual blood and marrow cells by immunofluorescence microscopy and of cultured chicken lymphoblasts by immunoblotting revealed that the beta tubulin variant was expressed in just erythrocytes and thrombocytes and their precursors. Examination of nine chicken tissues by immunoblotting revealed only small amounts of the erythrocyte tubulin variant, these amounts being consistent with the amounts expected for erythrocyte contamination in the tissues. It is interesting that erythrocytes and thrombocytes, which both contain the beta tubulin variant, are developmentally related and both contain a marginal band (Lucas and Jamroz, 1961). We presume that the expression of this unique tubulin is related to its special function in marginal band formation and maintenance, but this remains to be demonstrated.

There are several cases that clearly document the differential localization of tubulin isoforms, generated by posttranslational modifications or by differential gene expression, in different cell and tissue types. For example, tyrosinolated alpha tubulin has been localized in a specific subset of microtubules during interphase and in astral microtubules in dividing cells (Gunderson et al., 1984), and acetylated alpha tubulin has been determined to be specifically contained in axonemal microtubules in Chlamydomonas (L'Hernault and Rosenbaum, 1985). Specific beta tubulin genes are known to be expressed in Drosophila, chicken, and mouse. Of four distinct beta tubulin genes in chicken, one is predominantly expressed in brain and another in testis (Sullivan et al., 1984; Havercroft and Cleveland, 1984). Of the four beta tubulin genes in
Drosophila, one appears to be testis-specific (Kemphues et al., 1979, 1980) and another is expressed in embryonic tissues (Raff et al., 1982). The reasons for the specificity of expression of these tubulin gene products is in most cases unclear. Taken together, these observations support the hypothesis that different tubulin variants may perform different functions in the cell (Stephens, 1975; Fulton and Simpson, 1976).

At the present time the relationship between the expression of the erythrocyte beta tubulin variant and red cell differentiation is only partially understood. However, in vitro studies of erythrocyte tubulin assembly have begun to provide some insights. Like the marginal band microtubules in erythrocytes, purified erythrocyte tubulin has been found to be stable and to produce long microtubules. This has been found to be due to a low rate of nucleation which is in turn determined by a low critical concentration and to the formation of stable tubulin oligomers (Murphy and Wallis, 1985a, b; Rothwell et al., 1985a, b). Studies of erythrocyte tubulin assembly may provide yet additional insights on the possible requirement of this tubulin variant for marginal band formation.

**Differential Tubulin Gene Expression in Erythrocyte Development?**

We observed that the erythrocyte beta tubulin variant is not present in early stage erythroblasts and that this protein is suddenly expressed in midstage erythroblasts at the onset of marginal band formation. Although Northern blot analysis of early stage erythroblasts in early chick embryos by Havercroft and Cleveland (1984) revealed two different beta tubulin mRNAs (beta 3 and beta 4'), it is likely that the tubulins produced by these messages represent constitutive tubulins of early erythrocyte precursors and not the tubulin found in mature erythrocytes. Whereas Havercroft et al. observed that lymphocytes contain the same beta tubulin mRNA species, we failed to detect erythrocyte beta tubulin in the same cells by immunoblotting and immunofluorescence microscopy. Diversities in amino acid sequence (unpublished) and composition of erythrocyte beta tubulin (Murphy et al., 1984) also indicate that the erythrocyte beta subunit probably represents the product of a distinct beta tubulin gene that has not yet been identified and sequenced. Whether the sudden appearance of erythrocyte beta tubulin and marginal band formation in erythroblasts is associated with the activation of a unique tubulin gene remains to be determined.

**Marginal Band Formation**

The sequence of events in marginal band assembly suggests a complex process requiring the coordinated activity of several discrete events: (a) Reduction in cell size and nuclear diameter; (b) initiation at the centrosome of new microtubules containing erythrocyte variant and elongation of these microtubules into a dense wreath of individual microtubules; (c) subsequent loss of microtubule-organizing activity by the centrosome and consolidation of microtubules (perhaps by inter-tubule cross-bridging?) into the definitive marginal band. It is possible that this process is mediated by microtubule-associated proteins such as MAP-2 (Sloboda and Dickersin, 1980) and/or tau (Murphy and Wallis, 1985a) that are known to be contained in chicken erythrocyte marginal bands; and (d) association and attachment of the microtubule bundle to the cell membrane. The association of marginal band microtubules with the cell membrane can be seen directly by electron microscopy of thin sections, and has been observed indirectly, as in the reassembly of microtubules in extracted erythrocyte ghosts from which microtubules have been removed (Miller and Solomon, 1984; Swan and Solomon, 1984).

The fate of the pre-existing microtubules and centroosomes present in early erythroblasts is not known. Since centrioles have never been detected in chicken marginal bands, marginal band development in the chicken may be distinct from band formation in blood clot erythrocytes, where the centroes located at the cell periphery appear to act as nucleating centers for the elongation of marginal band microtubules (Nemhauser et al., 1983).

These observations establish that a biochemically distinct variant of beta tubulin is associated with erythrocytes and thrombocytes in chicken blood. It remains to be proved, however, whether this association is specific for these two blood cell types, or whether the erythrocyte tubulin variant is required for marginal band formation. The presence of microtubules with an apparent static and cytoskeletal function in these differentiated cells raises interesting questions regarding the dynamics of erythrocyte microtubules and the possible sorting of erythrocyte and constitutive tubulin subunits during marginal band formation. Further work will be required to explore the dynamics and biological function of these interesting polymers.

This work was supported by National Institutes of Health (NIH) Grants GM-26155 and GM-33171 and by an NIH Research Career Development Award CA-00645.

Received for publication 8 July 1985, and in revised form 14 October 1985.

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