Adducin in Erythrocyte Precursor Cells of Rats and Humans: Expression and Compartmentalization

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Adducin is a calmodulin-binding protein involved in the assembly of the erythrocyte membrane skeleton. To investigate the expression of adducin during human erythropoiesis, we performed immunofluorescence studies on smears of cultured human erythroblasts. Adducin immunoreactivity was found in the early stages of erythropoiesis. Proerythroblasts were the first erythroid precursor cells positive for adducin. The adducin signal was very similar to the signal of erythroid β-spectrin in that both proteins lined the membrane of erythroid precursor cells. Cell fractionation experiments were performed to further analyze the intracellular distribution of adducin in erythroid cells. In erythroblasts, about 60% of total cellular adducin appeared in the Triton-soluble fraction. In reticulocytes, the Triton-soluble fraction decreased to 30% of total reticulocyte adducin. Erythrocytes had no detectable amount of adducin in the Triton-soluble pool. Instead, adducin was quantitatively bound to the Triton-insoluble erythrocyte cytoskeleton. Our results suggest that adducin is expressed early in the development of the erythrocyte membrane skeleton, whereas stable assembly onto the membrane skeleton does not occur before the final stages of mammalian erythropoiesis.

In this study, we present evidence that, in mammalian erythropoiesis, adducin is an early expressed protein switching during erythrocyte maturation from a Triton-soluble pool to binding sites on the membrane cytoskeleton.

MATERIALS AND METHODS

Human bone marrow (BM) cell culture. Human BM was obtained from patients undergoing hip surgery. Spongiosa pieces were placed in phosphate-buffered saline (PBS; 10 mmol/L Na phosphate, 140 mmol/L NaCl, pH 7.4) anticoagulated with heparine (10 U/mL). After mechanically dissecting the spongiosa, the cells were filtered through a 100-μm nylon mesh. The resulting cell suspension was loaded on a Ficoll-gradient (Pharmacia, Freiburg, Germany) and centrifuged for 20 minutes at 800g. The interphase of the Ficoll gradient was seeded on fibronectin-coated glass slides at a density of 5 × 10^6 cells/mL. Erythroblasts were grown in Iscove's medium (GIBCO, Eggenstein, Germany) supplemented with 5 × 10^{-4} mol/L hydrocortisone (Sigma Chemie, Deisenhofen, Germany), 0.1 mmol/L α-thioglycerol (Sigma), 3 U/mL human recombinant erythropoietin (Epo), 50 ng/mL human recombinant interleukin-3 (IL-3), and 25% fetal calf serum (FCS; GHBCO) in a humidified atmosphere with 12.5% O_2 and 5% CO_2 at 37°C. Epo and IL-3 were kind gifts of the Behringwerke (Marburg, Germany).

For immunofluorescence, air-dried smears of BM cells were fixed in 100% methanol for 3 minutes at 20°C and processed for immunofluorescence with 5% CO_2 for 3 minutes at 20°C and processed for immunofluorescence according to standard procedures.

Treatment of animals. Anemia was induced in Wistar rats by intraperitoneally injecting 0.5 mL/d of 1.5% wt/vol phenylhydrazine hydrochloride (Sigma) in 50 mmol/L Tris, pH 7.4, over a range of 5 days. Two days after the last injection, rats were anesthetized with pentobarbital and killed by decapitation. Spleens were collected in Hanks' solution without Ca^{2+} and Mg^{2+} (Sigma) with 10% FCS added, and blood was collected in PBS with heparin.

Isolation of nucleated red blood cell (RBC) precursors and reticulocytes. RBC precursors were isolated essentially as described by Hanspal and Palek with the modification that spleens were minced in Hanks' solution with 10% FCS added to avoid agglutination of precursor cells. Erythroblasts were identified by Pappenheim staining and reticulocytes by methylene blue staining. Viability was evaluated by the trypsin blue exclusion test. Fractions containing greater than 80% erythroblasts and greater than 90% reticulocytes, respectively, were used for the experiments. Erythrocytes were
collected from healthy control rats and washed threefold in PBS to remove theuffy coat.

**Antibodies and proteins.** RBC adducin was purified from outdated human erythrocyte concentrates essentially as previously described, with the modification that sedimentation on a sucrose gradient was substituted by Mono S-chromatography. Purified adducin was digested with trypsin (3 μg trypsin/mg adducin for 30 minutes at 4°C). The digest was passed over a Mono Q column (Pharmacia, Uppsala, Sweden) and a protease-resistant domain was isolated. Total antiadducin antiserum from rabbits was affinity purified using the protease-resistant domain of adducin coupled to cyanogen bromide-activated sepharose 4 B (Pharmacia). A second adducin antibody was raised in rabbits by injection of purified native adducin. In immunoblots, both adducin antibodies reacted strongly with α- and β-adducin and, additionally, with a breakdown product of about 70 Kd. This 70-Kd band appeared as a double-band in most preparations. By immunoblotting, the antibody directed to the total adducin molecule showed weak binding to a protein band of about 200 to 220 Kd (see Fig 1). Because this band could not be abolished by affinity purification of the total adducin antiserum on nitrocellulose-bound purified adducin, it was probably caused by dimerization of adducin monomers. For immunofluorescence purposes, we used the adducin antibody directed to the protease-resistant domain of adducin. For immunoblotting purposes, we used the total adducin antibody. The rabbit antibody directed to erythrocyte β-spectrin was affinity purified on purified β-spectrin coupled to cyanogen bromide-activated sepharose 4 B. By immunoblotting, the antibody reacted strongly with β-spectrin without showing cross-reactivity to other proteins.

**Subcellular fractionation of RBC precursors.** To assess the subcellular distribution of adducin, erythroblasts, reticulocytes, and mature erythrocytes were lysed separately in 1% Triton X-100 (Sigma) in 10 mmol/L Tris, pH 7.2, 150 mmol/L NaCl, 5 mmol/L MgCl₂, 2 mmol/L Na EDTA, and 0.25 mmol/L dithiothreitol (DTT; Sigma). The lysis buffer always contained protease inhibitors diisopropylfluorophosphat (DFP; 0.5 μmol/L; Sigma), pepstatin (1 μg/mL; Sigma), leupeptin (1 μg/mL; Sigma), and phenylmethylsulfonylfluoride (PMSF; 20 μg/mL; Sigma). All fractionation steps were performed at 4°C. Nuclei were removed by centrifugation at 800g for 5 minutes. The Triton-resistant cytoskeletons were pelleted by centrifugation at 40,000g for 20 minutes and washed once in excess lysis buffer. Pellets were lysed in sodium dodecyl sulfate (SDS)-sample buffer and the Triton-soluble supernatants were precipitated in 70% ethanol at -20°C before dissolution in SDS-sample buffer. Protein determination was performed on proteins dissolved in SDS-sample buffer by the method of Heinz et al. Equal amounts of protein were loaded on SDS-Laemmli gels, separated electrophoretically and transferred to nitrocellulose using standard procedures. Immunoblotting with antiadducin antibody was performed and bound IgG was visualized with 125I-protein A and quantitated by gamma-scanning with a linear analyzer. The Triton-soluble adducin was related to total cellular adducin and expressed as the percentage of total cellular adducin.

**RESULTS**

**Immunofluorescence of human erythroblasts.** In addition to myeloblastic cells and macrophages, the BM culture used for the immunofluorescence experiments contained about 50% RBC precursors ranging from proerythroblasts to completely enucleated reticulocytes. All developmental stages were distinctly labeled with adducin antibodies (Fig 1).
Fig 2. Air-dried smears of cultured human BM cells. (a) Pappenhein staining. pb, proerythroblast; n, group of normoblasts; mm, metamyelocyte; mc, myelocyte. (b) Nonimmune serum control; note the unspecific fluorescence of myelocytic granules (m) (bar = 10 μm). (c) Immunofluorescence with an antibody to human adducin. eb, erythroblast; n, normoblasts of different developmental stages; m, myeloid cell, displaying weak and diffuse staining (compare with myeloid cell in d). (d) Immunofluorescence with an antibody to human RBC β-spectrin. pb, proerythroblasts; n, normoblast; m, myeloid cell that stains unspecifically due to granule content.

2). Proerythroblasts already were positive for adducin and exhibited a fluorescence pattern that was partially located on the membrane. The confinement of the label to the membrane increased with ongoing maturation and appeared almost complete in normoblasts (Figs 2 and 3). Conversely, the intensity of the label that stained the perinuclear region and preferentially the Golgi area of proerythroblasts and early erythroblasts decreased during RBC development. In myeloblasts and macrophages, the adducin label was not confined to the membrane. Rather, these cells displayed a weak and diffuse perinuclear staining (Fig 2c). Immunofluorescence with an antibody specific for erythroid β-spectrin resulted in a label very similar to that seen with antiadducin. Again, erythroid cells of all

**ADDUCIN**

**β-SPECTRIN**

Fig 3. Sequence of successive developmental stages of cultured human erythroblasts as visualized by immunofluorescence with antibodies against adducin and RBC β-spectrin. 1, proerythroblast; 2, erythroblast; 3, polychromatophilic normoblast; 4, orthochromatophilic normoblast; 5, mature human erythrocytes.
developmental stages were strongly labeled and the fluorescence was found restricted to the membrane. However, nonerythroid cells did not react with the β-spectrin antibody (Fig 2d). The close resemblance between the adducin label and the label for β-spectrin is depicted in Fig 3, which shows the major developmental steps of human erythropoiesis as visualized by immunofluorescence with antibodies to adducin and to erythroid β-spectrin. The fluorescence is partially confined to the membranes of proerythroblasts, whereas another part stains the cell pole, which is facing the nucleus. This cellular region is well known for containing the internal membranes of the Golgi apparatus and the endoplasmic reticulum. When maturation progresses, an increasing amount of label is found on the outer cell membrane and a decreasing amount perinuclearly. Or-thochromatophilic normoblasts (Fig 3, panel 4) and reticu-locytes (not shown) show a “pseudocytoplasmic” staining that is due to membrane ruffling and thus superimposition of membrane-bound fluorescence.

Subcellular fractionation of RBC precursors. For biochemistry purposes, the human erythroblast culture used for the immunofluorescence experiments neither provided enough material nor fractions containing only erythroid progenitor cells. Therefore, we decided to study erythroblasts in spleens of phenylhydrazine-treated rats. Erythroblasts and reticulocytes were isolated from phenylhydrazine-treated anemic rats and compared with mature erythrocytes from healthy controls. Highly enriched fractions of erythroblasts, reticulocytes, or erythrocytes were lysed separately in an isotonic buffer with 1% Triton X-100, and Triton-soluble and Triton-insoluble fractions were prepared (Fig 1).

Scanning of 125I-protein A-labeled immunoblots showed that about 60% of total erythroblast adducin is in a Triton-soluble state (Fig 1B). This percentage decreased to 30% in reticulocytes. Erythrocytes did not contain any detectable amounts of adducin in their Triton-soluble supernatant. Instead, erythrocyte adducin was quantitatively bound to the detergent-nonsoluble cytoskeleton. The comparison of cytoskeletal fractions showed that reticulocytes still lack 20% of adducin on their membranes, whereas erythroblasts have a deficit of at least 70% (Fig 1C). The actual value for the erythroblast cytoskeleton may even be lower, because in our erythroblast fractions we counted 15% to 20% reticulocytes, which are supposed to contribute considerably to the cytoskeleton-bound adducin of the erythroblast fraction. By measuring the amount of adducin in total cells, we found nucleated precursor cells to be provided with a full amount of adducin (Fig 1A). In some preparations we even determined that the amount of adducin in erythroblasts is 1.5-fold higher than in reticu-locytes.

DISCUSSION

We provided evidence that, in human erythropoiesis, the cytoskeletal protein adducin is an early expressed protein. Adducin is already present in proerythroblasts and is found lining the membrane of the precursor cells. With regard to these features, the fluorescence pattern for RBC β-spectrin was almost undistinguishable from that shown for adducin. Subcellular fractionation experiments showed that about 60% of erythroblast adducin was localized in the Triton-soluble pool. Considering that the erythroblast fraction used for the fractionation experiments represented an inhomogeneous cell population ranging from proerythroblasts to normoblasts and even reticulocytes, it is reasonable to assume that the percentage of Triton-soluble adducin is even higher in immature developmental stages as compared with normoblasts. Thus, we conclude that adducin associates loosely with the membrane skeleton of erythrocyte precursors and that this binding is gradually strengthened in the course of erythrocyte maturation. Alternatively, the spectrin skeleton has not yet assembled to the point that the structure remains intact after extraction with detergent. Stabilization of the membrane skeleton obviously has not yet finished with the enucleation event of normoblasts because reticulocytes still had an incomplete amount of adducin on their membrane and about 30% of total reticulocyte adducin was found in the Triton-soluble pool. Furthermore, metabolic labeling experiments suggested that the fraction of adducin that assembles onto the reticulocyte membrane has been synthesized in earlier stages of development because we could show synthesis of adducin only for erythroblasts but not for reticulocytes (not shown). In conclusion, these data seem consistent with the assumption that, despite early expression, adducin contributes to the stabilization of the erythrocyte membrane during the final phase of erythropoiesis.

REFERENCES

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