Proc. Natl. Acad. Sci. USA
Vol. 74, No. 9, pp. 3898–3902, September 1977
Cell Biology

Induction of spectrin in erythroleukemic cells transformed by Friend virus

(Friend erythroleukemia cells/erythrocyte membrane/phosphorylation)

H. EISEN, R. BACH, AND R. EMERY

Department of Molecular Biology, University of Geneva, Geneva, Switzerland

Communicated by François Jacob, June 28, 1977

ABSTRACT **The presence and accumulation in murine erythroleukemic cells transformed by Friend virus of the erythrocyte membrane-associated protein spectrin has been investigated. Spectrin was present in the uninduced cells and was induced 10- to 20-fold in dimethyl sulfoxide-treated differentiating cells. The intracellular concentration of spectrin reached a peak on the third day of dimethyl sulfoxide treatment, after which it fell to levels found in mouse erythrocytes. We also found that the small subunit of spectrin was phosphorylated in the cells.**

Erythroleukemic cells transformed by Friend virus (FL cells) have become a useful experimental system for *in vitro* study of the later stages of erythroid differentiation (1–3). While untreated FL cells show few erythroid characteristics, addition of many different substances to the cells induces what resembles normal erythroid differentiation, resulting in the appearance of globin mRNA and globin in the cytoplasm (4, 5), the decrease in the expression of the major histocompatibility antigen, H-2 (6) on the cell surface, the appearance of erythroid antigens (7), and the arrest of cell division and nuclear activity (8). In an attempt to understand the behavior and role of the cell membrane in this erythroid differentiation, we have studied the induction and metabolism of the erythrocyte membrane-associated protein spectrin.

Spectrin comprises approximately 25% of the protein of the erythrocyte membrane (9, 10). This protein, which is composed of two subunits of molecular weights of 240,000 (band 1) and 220,000 (band 2), is associated with the inner surface of the plasma membrane and appears to interact with actin (11, 12). The role of spectrin within the erythrocyte is not understood. It is thought to influence the state of the cell membrane in that its presence is associated with a lack of lateral mobility of membrane proteins and intramembranous particles (13). The smaller subunit of spectrin is phosphorylated *in vivo* (14), and the degree of phosphorylation has been suggested to play a role in the regulation of cell shape (15). Little has been done to examine the presence and role of spectrin in nucleated erythroid precursors, presumably because pure populations of such cells are difficult to obtain. The FL cells provide a useful experimental system for such studies.

MATERIALS AND METHODS

Cells and Culture Conditions. FL cells of clone F4N and P815 mastocytoma cells were obtained from W. Ostertag and P. Vassalli, respectively. The cells were cultivated as described (16). Bone marrow cells were extruded from the femurs into cold Earle's salts, and suspensions were obtained by pipetting. The cells were washed three times in cold Earle's salts before use. Fetal livers were taken from 12- to 13-day fetuses of Swiss mice. Cell suspensions were prepared as described for bone marrow cells. Hemoglobin-containing cells were detected by the wet benzidine method (17).

Reagents. Spectrin was purified from mouse erythrocyte ghosts by the method of Marchesi (18), and was iodinated by the method of McConahey and Dixon (19). Antiserum against mouse spectrin was obtained from rabbits after two intradermal injections of 1 mg of purified protein in complete Freund's adjuvant at 6-week intervals followed by two or more intramuscular injections of 1.5 mg of purified protein at 2-week intervals. The IgG fractions of sera were prepared by the method of Harboe and Ingild (20). Sheep antiserum against rabbit IgG (SaRIg) was obtained from P. Vassalli, and the rhodamin-coupled IgG fraction was prepared by the method of Lamelin *et al.* (21). *Staphylococcus aureus* was grown and prepared by the method of Kessler (22).

Fluorescent Staining. Washed cells to be stained were suspended at a cell density of 5×10^5 cells per ml in Earle's salts containing 10 mg of bovine serum albumin per ml. They were then put on microscope slides by cytocentrifugation, air dried, fixed for 5 min at −20° in either methanol or acetone, and immediately placed in cold phosphate-buffered saline. Indirect staining was used for detection of spectrin. The fixed cells were covered with a drop of the IgG fraction of rabbit antiserum to mouse spectrin diluted in Earle's salts containing 5% bovine serum albumin and left for 20 min at room temperature. After the cells were washed in phosphate-buffered saline, they were covered with a drop of rhodamine-conjugated SaRIg diluted in Earle's salts containing 5% bovine serum albumin and left for 20 min at room temperature. After the cells were washed in phosphate-buffered saline followed by H_2O, the cells were examined under glycerol containing 5% phosphate-buffered saline in a Zeiss fluorescence microscope.

Radioimmune Assay. Cells to be examined (10^7) were washed in phosphate-buffered saline and once in buffer A (10 mM Tris·HCl, pH 8.0/1 mM EDTA/0.34 M sucrose) and resuspended in 0.1 ml of the same buffer containing 1 mM ATP, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride. The cells were lysed by addition of 1% Nonidet P-40 and sonicated for 1 min, after which 0.4 ml of buffer B (10 mM Tris·HCl, pH 8.0/0.5 M NaCl/2% 2-mercaptoethanol) was added. Immune precipitation was carried out in 0.1 ml of buffer B containing 0.1% sodium dodecyl sulfate ($NaDodSO_4$). Each reaction mixture contained 5 ng of ^{125}I-labeled mouse spectrin (1 to 2×10^4 cpm/ng), 10 μl of a 1:1600 dilution of rabbit antiserum to mouse spectrin diluted in pre-immune rabbit serum, and 10

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Abbreviations: FL cells, murine erythroleukemia cells transformed by Friend virus; Me_2SO, dimethyl sulfoxide; $NaDodSO_4$, sodium dodecyl sulfate; SaRIg, sheep antiserum to rabbit IgG.

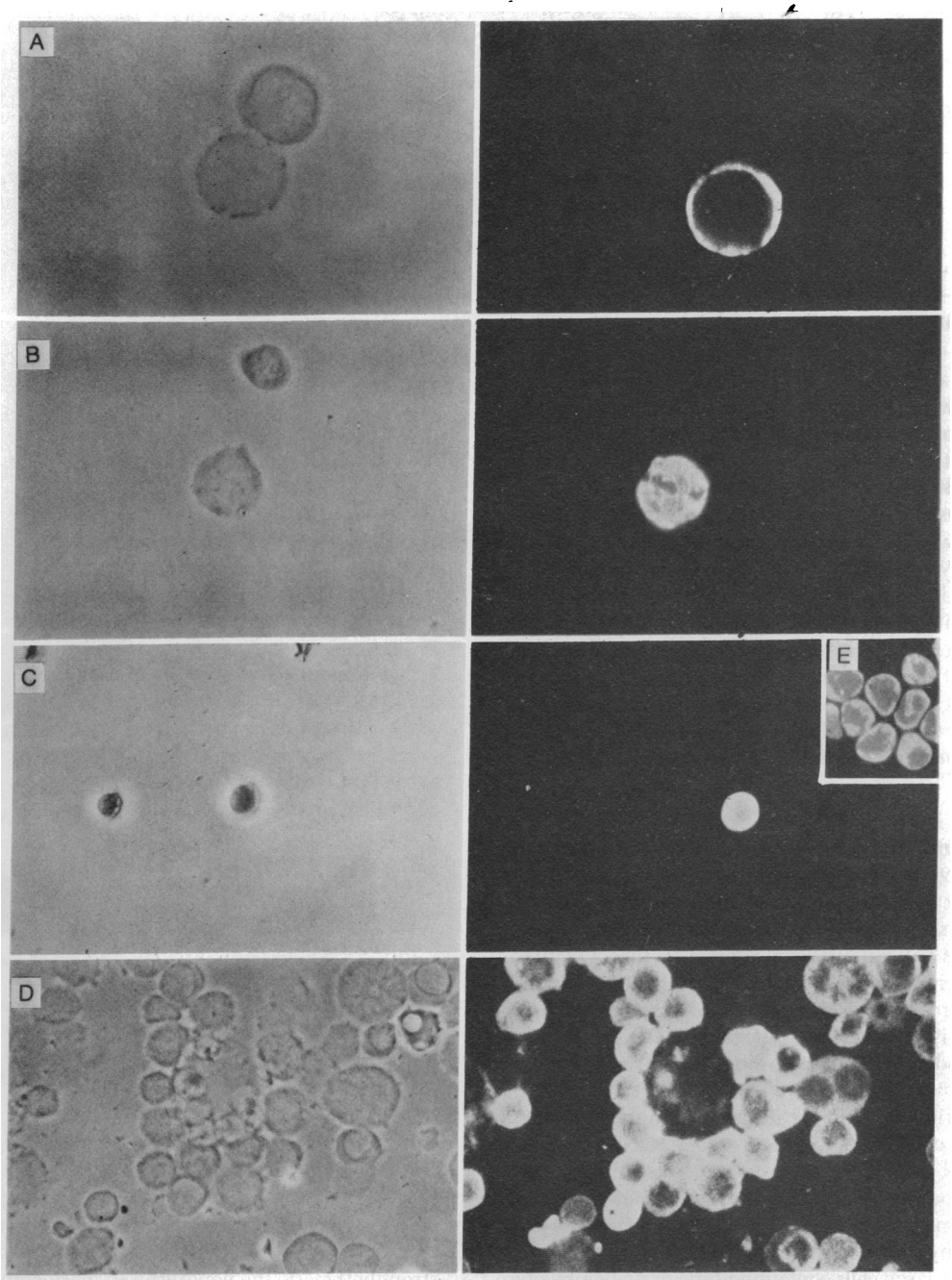

FIG. 1. Fluorescent antibody staining of mouse erythrocytes and spectrin-containing nucleated cells of mouse bone marrow and fetal liver. (*A*, *B*, and *C*) Mouse bone marrow cells stained with antiserum to spectrin at a final dilution of 1/80. Photographs in the left panels were taken with phase contrast optics; those in the right panels were taken with fluorescence; 3-min exposure. (×750.) (*D*) Rosette of developing erythroid cells from mouse 12-day fetal liver stained with antiserum to spectrin at a final dilution of 1/80; 3-min exposure. (×390.) (*E*) Mouse peripheral erythrocytes stained with antiserum to spectrin at a final dilution of 1/40; 6-min exposure. (×700.)

μl of either standard solutions of purified mouse spectrin or cell extracts. The mixtures were incubated for 12–15 hr at 4°, after which 0.2 ml of a 10% (vol/vol) suspension of treated *S. Aureus* was added and incubation was continued for 30 min at 0°. The bacteria were then centrifuged and washed three times in ice-cold buffer C (10 mM Tris·HCl, pH 8.0/1 mM EDTA/2% 2-mercaptoethanol/1% Nonidet P-40/10% glycerol/50 μg of toluenesulfonylfluoride per ml) and the bound radioactivity was determined. Protein concentrations were determined by the method of Lowry *et al.* (23), with bovine serum albumin as a standard.

Immune Precipitation. After incubation in $^{32}PO_4$ or [^{35}S]methionine, the cells were washed and the spectrin was extracted as described above for the radioimmune assay. The extracts were centrifuged at 100,000 × *g* for 30 min and the supernatant was kept. To 150 μl of 100,000 × *g* supernatant were added 5 μg of the IgG fraction of rabbit antiserum to spectrin, and the mixtures were incubated at 4° for 15 hr. An equivalent amount of the IgG fraction of SaRIg was then added and incubation was continued for a further 12–15 hr at 4°. The precipitates were then washed three times in buffer C and finally dissolved in 80 μl of sample buffer (24). The entire samples were applied to 5% $NaDodSO_4$/polyacrylamide slab gels prepared according to Laemmli (24) and analyzed by electrophoresis. The gels were then stained as described previously (16), dried, and autoradiographed.

Table 1. Staining of FL cells for the presence of spectrin and hemoglobin*

Time in 1.25% Me_2SO, hr	% positive FL cells	
	Spectrin	Benzidine
0	0.3	0.1
24	4	0.5
48	38	17
72	87	56
96	92	81

* Treatment of mastocytoma cells with Me_2SO did not induce spectrin or hemoglobin.

Measurement of Spectrin and Globin Synthetic Rates. Cells (2.5×10^6) were incubated for 1 hr in the presence of 50 μCi of [^{35}S]methionine. They were then washed and extracted as described for the radioimmune assay and centrifuged at $100,000 \times g$ for 30 min. A portion of the supernatant was diluted to 20% of its original concentration with sample buffer and the proteins were analyzed by electrophoresis in 10% $NaDodSO_4$/polyacrylamide slab gels. The gels were dried and autoradiographed and autoradiographs were scanned with a Joyce Loebl densitometer. The remainder of each sample was analyzed for spectrin by immune precipitation as described above. After electrophoresis and autoradiography the autoradiographs were scanned. The areas under the peaks of globin and spectrin were measured. The results were corrected for the amount of acid-insoluble radioactivity in the extracts.

RESULTS

Fluorescent Antibody Staining. In order to examine for the presence of spectrin-containing cells, rabbit antiserum was prepared against purified mouse erythrocyte spectrin. The antibodies were shown to be specific for spectrin by immunoelectrophoresis and gel diffusion against erythrocyte ghosts and immune precipitation of extracts of [^{35}S]methionine-labeled mouse bone marrow cells followed by $NaDodSO_4$/polyacrylamide gel analysis. Absorption of the serum with spectrin purified by $NaDodSO_4$/polyacrylamide gel chromatography completely removed the activity. The IgG fraction of the antiserum to spectrin was used for indirect immunofluorescent staining of fixed cells.

The staining of normal mouse bone marrow cells, fetal liver cells, and erythrocytes by anti-spectrin IgG is shown in Fig. 1. In normal bone marrow between 18 and 25% of the nucleated cells are stained by the antibodies. These spectrin-positive cells range from rather large cells containing little or no hemoglobin, as measured by Soret band microscopy (25), to very small normoblast-like cells as well as reticulocytes and erythrocytes. Erythrocytes (Fig. 1*E*) appear to stain less brightly than do their nucleated precursors (the concentration of antiserum to spectrin used to stain the erythrocytes was twice that used for the nucleated bone marrow cells). Fig. 1*D* shows a photograph of fluorescent fetal liver cells stained with anti-spectrin IgG, which are 80–90% erythroid. The photograph represents a large cell (presumably a histiocyte) surrounded by developing spectrin-positive cells. Such erythroid rosettes are extremely common and may represent the organization of erythropoiesis in the fetal liver (26).

FL cells of clone F4N were examined for the presence of spectrin by indirect immunofluorescent staining before induction and at 24-hr intervals after the addition of 1.25% dimethyl sulfoxide (Me_2SO). Both the uninduced and induced FL cells were compared with nonerythroid cells of the DBA/2 mastocytoma P815. The numbers of hemoglobin-containing

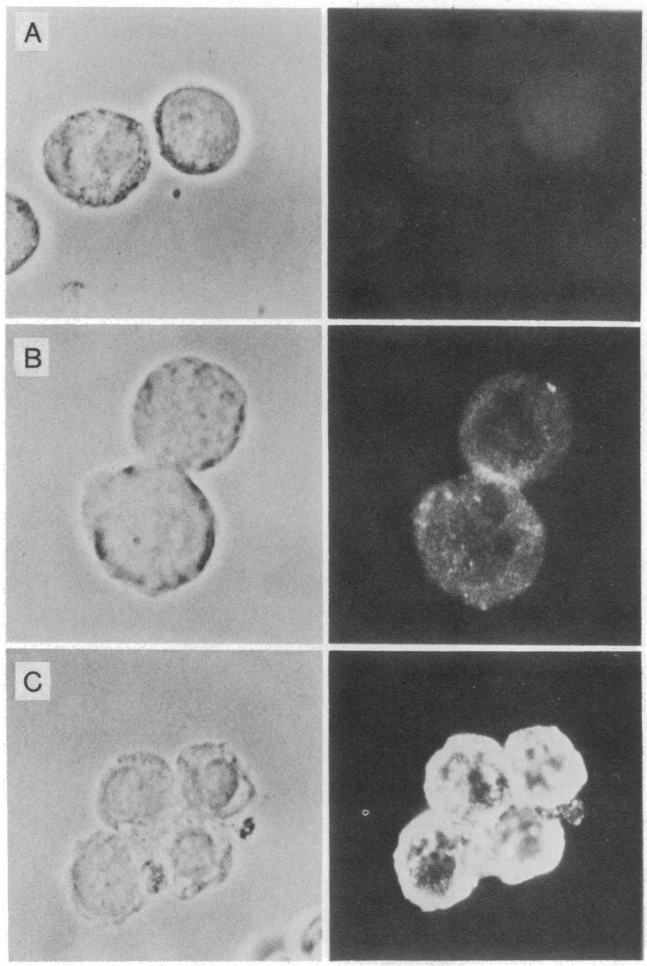

FIG. 2. Fluorescent antibody staining of mastocytoma and FL cells. The left panels represent the cells seen in the phase contrast, while the right panels represent the same fields in fluorescence. In all cases antiserum to spectrin was used at a final dilution of 1/80 and exposure of the film to fluorescence was for 3 min. (×1200.) (*A*) mastocytoma; (*B*) FL cells, untreated; (*C*) FL cells (clone F4N) induced for 3 days with 1.25% Me_2SO.

cells in the populations were measured by benzidine staining as a separate indication of the degree of erythroid differentiation. The results are shown in Table 1 and Fig. 2. Although there was very weak fluorescent staining in the uninduced FL cells (0.2–0.5% of the cells stained brightly), these cells still stain more strongly than do the nonerythroid mastocytoma cells. Addition of Me_2SO to the FL cells resulted in an increase in the number of brightly staining spectrin-positive cells, with the number of positive cells reaching a maximum after 72 hr. The appearance of spectrin-positive cells consistently preceded the appearance of hemoglobin (benzidine)-positive cells (see below). Identical results were obtained with other FL cell inducers such as hexamethylenebisacetamide (27) and hypoxanthine (28). Treatment of the mastocytoma cells with FL cell inducers had no effect on the induction of either spectrin or hemoglobin.

Radioimmune Assay. In order to quantitate the appearance of spectrin during induced differentiation of the FL cells, we have developed a radioimmune assay. Cells of clone F4N were stimulated to differentiate by addition of 1.25% Me_2SO. At 24-hr intervals samples were removed and treated and the spectrin content was determined. It may be seen in Fig. 3 that the uninduced FL cells contain some spectrin (0.015% of the cell protein) while none was detected in the control mastocytoma cells. This is in agreement with the results observed by

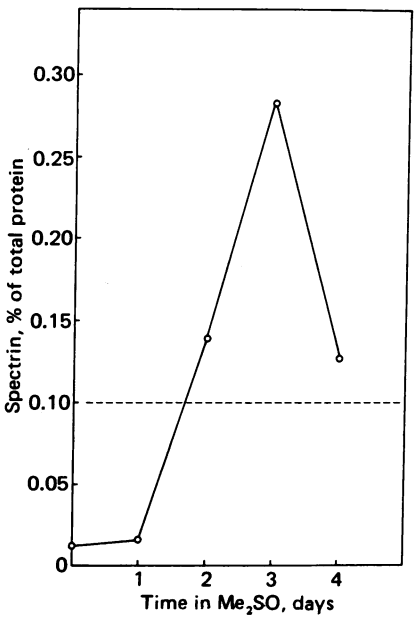

FIG. 3. Appearance of spectrin in FL cells (clone F4N) during treatment with 1.25% Me_2SO. The dashed line represents the percent of spectrin in normal mouse erythrocytes. The solid line represents the FL cells.

fluorescence microscopy. While there is little increase in the specific activity of spectrin during the first 24 hr of Me_2SO treatment, the specific activity begins to rise after that time, reaching a maximum on day 3, when it is 10- to 20-fold higher than that of the uninduced cells. On the fourth day of Me_2SO treatment the specific activity of spectrin in the induced cells fell to approximately the same level as that found in mouse erythrocytes. This decrease in spectrin content of individual FL cells was also observed when fluorescent antibody staining was used. We have also observed that the more mature erythroid cells in normal mouse bone marrow (reticulocytes) appear to contain less spectrin than the immature cells (Fig. 1). In this case the decrease in spectrin content of the cells appears to accompany loss of the cell nucleus.

The finding that in the induced FL cells spectrin appeared earlier than hemoglobin and that its accumulation stopped before that of hemoglobin prompted us to compare its rate of

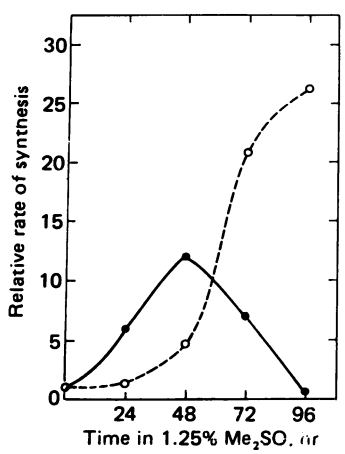

FIG. 4. Synthesis of spectrin and globin during Me_2SO treatment of FL cells. Synthesis of globin and spectrin was measured. The rates of synthesis of the proteins are plotted in arbitrary units relative to the rates of synthesis found in untreated cells. ●, Spectrin; ○, globin.

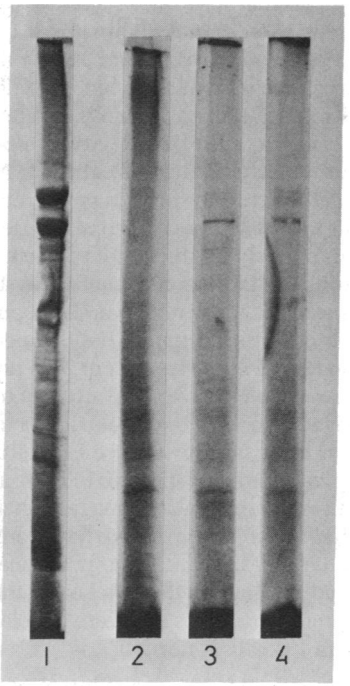

FIG. 5. Analysis of immune precipitates of spectrin from ^{32}P-labeled FL cells on $NaDodSO_4$/polyacrylamide gels. Lane 1, mouse reticulocyte ghosts stained with Coomassie blue. Lane 2, ^{32}P-labeled uninduced FL cells immune precipitated with pre-immune rabbit IgG. Lanes 3 and 4, the immune precipitates of ^{32}P-labeled FL cells treated for 0 and 2 days, respectively, with 1.25% Me_2SO and precipitated with rabbit anti-mouse spectrin IgG.

synthesis with that of globin. Samples of FL cells taken at various times after addition of 1.25% Me_2SO were incubated for 1 hr with [^{35}S]methionine, washed, and extracted. The extracts were then analyzed for radioactive globin and spectrin, and the relative rate of synthesis of each was calculated. The results are shown in Fig. 4. The rate of synthesis of spectrin increased 5-fold by 24 hr and reached a maximum by 48 hr, after which it dropped sharply. The increase in the rate of globin synthesis occurred later than that of spectrin synthesis and continued to rise until the end of the experiment.

Phosphorylation of Spectrin in FL Cells. We have also examined the state of phosphorylation of spectrin in uninduced and induced FL cells of clone F4N. Cells (10^7) were incubated for 2 hr in 10 ml of PO_4-free medium containing 0.1 mCi of $^{32}PO_4$ per ml. They were then washed and lysed. The spectrin was then immunoprecipitated and analyzed by electrophoresis in a $NaDodSO_4$/polyacrylamide gel. The gel was dried and autoradiographed. The results are shown in Fig. 5. Pre-immune rabbit IgG did not precipitate any labeled proteins corresponding to either of the bands of spectrin. However, rabbit anti-spectrin IgG precipitated a band from both uninduced and induced FL cells which migrated with band 2 of erythrocyte spectrin. It thus appears that band 2 of spectrin is phosphorylated in both uninduced and induced FL cells.

DISCUSSION

We have demonstrated that uninduced FL cells contain the erythrocyte membrane-associated protein spectrin. Although spectrin is present in low levels in the uninduced FL cells (0.01–0.02% of the total cell protein), all of the cells contain it. We have also shown that Me_2SO treatment of the cells induces the synthesis of spectrin before that of globin and that the synthesis of spectrin is arrested before globin synthesis reaches

its peak. This suggests that spectrin is synthesized earlier during erythroid differentiation than is globin and is consistent with the finding of Chang *et al.* (29) that in the mouse *in vivo* spectrin synthesis precedes that of globin. Addition of inducers of erythroid differentiation to the cells resulted in a 10- to 20-fold increase in the specific activity of spectrin, which reached a maximum after 72 hr of treatment. After the third day of inducer treatment the spectrin content of the cells fell to a level similar to that found in normal mouse erythrocytes. The mechanisms underlying the loss of spectrin from the cells at late times during the induction are not understood. It cannot be due to uninduced cells growing up in the population as more than 90% of the cells in the population stained brightly for spectrin after 96 hr of Me_2SO treatment. It is possible that the loss of spectrin represents an abortive attempt to expel the FL cell nucleus. It is also possible that spectrin not localized in the cell membrane is lost from the mature FL cells.

The finding that band 2 of spectrin in both uninduced and induced FL cells is phosphorylated suggests that the protein is functional in these cells. However, we have found that induction of the FL cells results in the appearance of a form of spectrin not found in either erythrocytes or erythroid precursors (unpublished observations). It is therefore not clear whether the spectrin found in FL cells is normal.

This work was supported by a grant from the Swiss National Foundation for Scientific Research.

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