Synthesis and Phosphorylation of Plasma Membrane Proteins of Friend Erythroleukemia Cells Induced to Differentiate

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ABSTRACT

Friend virus-transformed murine erythroleukemia (FL) cells, either untreated or induced to differentiate by treatment with hexamethylene bisacetamide, were fractionated and subjected to polyacrylamide gel electrophoresis. Analysis of the fractions of the treated cells revealed no changes in either the rate of synthesis or the accumulation of the membrane proteins, including protein bands comigrating with spectrin, actin, and Band 3, as compared to the fractions of the untreated cells. Only a small fraction of a protein band comigrating with spectrin was associated with the plasma membrane. The bulk of the spectrin was detected in the cytosol cross-linked via S—S bonds to form aggregates. Protein bands comigrating with actin and Band 3 were distributed equally between the plasma membrane and the cytosol fractions.

However, differences between untreated and induced cells were observed in regard to the pattern of phosphorylation of the proteins. Dephosphorylation of the proteins of the differentiating cells, particularly those of the plasma membrane, could be detected shortly after treatment. Turnover of labeled phosphate in spectrin molecules from hexamethylene bisacetamide-induced, as well as uninduced, cells was very slow. Although actin from uninduced cells incorporated labeled phosphate very rapidly, in treated cells, the rate of incorporation decreased 24 hr after onset of differentiation. In addition, the plasma membrane proteins differed from those obtained from normal erythroid cells in several respects. (a) Turnover of phosphate in spectrin was more rapid in reticulocytes and mature erythrocytes as compared to the amount of label detected in the comigrating band of spectrin from induced or uninduced FL cells. (b) FL cell spectrin bands migrate with an apparent molecular weight of about 200,000 to 220,000, whereas normal erythrocyte spectrin bands migrate with a molecular weight of 200,000 to 240,000. And (c) Band 3, M.W. 100,000 protein, although comigrating with the mouse erythrocyte Band 3, was heterologous rather than homologous. These data suggest that the plasma membrane proteins of FL cells may be of an abnormal type.

INTRODUCTION

FL4 cells can be induced to differentiate by a variety of structurally unrelated chemical compounds, some of which are known to affect RNA and DNA synthesis (4, 9, 17, 31). Other inducers are typical membrane-active agents, such as ouabain, a specific inhibitor of Na+-K+-ATPase (2). Polar (20, 27) or cryoprotective (8, 26) compounds, which are in essence amphiphatic in nature, may also act on the plasma membrane. Most of the above-mentioned inducers cause early changes such as: (a) decrease in transport of small molecules (11, 16, 22); (b) early inhibition of phosphate metabolism and modification of phospholipid biosynthesis (17); and (c) early elevation of cyclic adenosine 3':5'-monophosphate level (11), suggesting that the differentiation process is triggered on the plasma membrane.

Since the phosphorylation level of plasma membrane proteins, including glycoproteins and membrane-bound enzymes, may play an important role in cell growth and differentiation, the effect of the inducers on the rate of synthesis and phosphorylation of the various plasma membrane proteins in FL cells was measured. The rate of accumulation in the various fractions of the cell of typical RBC markers such as spectrin, actin, and Band 3 was determined in view of differences in reports on the accumulation of RBC markers in the induced cells (5, 28).

In the present study, direct biochemical analysis of the various cell fractions revealed that an early decrease in the phosphorylation level of the plasma membrane proteins occurs in induced FL cells. However, the rate of synthesis and accumulation of plasma membrane protein bands tentatively identified as spectrin, actin, and Band 3 are similar in both induced and uninduced cells. Of particular interest was the finding that the properties of FL cell plasma membrane proteins differ in some respects from those of normal mouse erythrocyt cells.

MATERIALS AND METHODS

Cell Culture. FL cells, clone DS19, derived from clone 745, were cultured (1 × 106 cells/ml) in Eagle’s basal medium (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 15% (v/v) fetal calf serum (Grand Island Biological Co.) for 15 hr to allow one population doubling. HMBA was then added at the concentrations indicated in the text. After 72 hr, the cultures were stained by the method of Orkin et al. (24) to score hemoglobin-synthesizing, benzidine-positive cells. At this time, about 1% of the control cells and approximately 80% of the treated cells induced to differentiate were benzidine positive. Culture conditions for the Me2SO-resistant cell line DR10 (11) were the same. This line does not differentiate in the presence of Me2SO but is responsive to treatment with HMBA.

Cell Labeling. Cell cultures were sampled at the time points indicated in the text. The cells were spun down (100 × g for 5 min) and resuspended in prewarmed fresh complete medium.
to a final concentration of 2 to 3 x 10^6/ml. For methionine incorporation studies, [35S]methionine (New England Nuclear, Boston, Mass.; 500 Ci/mmol) was added to give a final specific activity of 200 μCi/μmol. The cells were harvested after a 3-hr pulse and washed 3 times in 0.9% NaCl solution. For phosphorylation studies, H_3PO_4 (New England Nuclear; carrier free) was added to give a final specific radioactivity of 50 μCi/μmol. The cells were harvested after a 3-hr pulse and washed 4 times in Eagle’s basal medium containing 10 mM phosphate buffer, pH 7.4.

Cell Fractionation. Cell pellets (2 to 4 x 10^7 cells) were lysed in 0.5 ml of ice-cold 5 mM Tris-HCl buffer, pH 7.4, containing 2.5 mM MgCl_2. Cell lysis was allowed to continue for 10 min in ice, after which the cells were spun down at 100 x g for 5 min. The supernatant was collected and further fractionated by centrifugation at 100,000 x g for 1 hr. Both the supernatant and the pellet of the cytosol fraction were kept.

The remaining “nucleated ghosts” were incubated for 5 min in 0.25 ml of 0.1% (v/v) NP40 in the same buffer in ice. At this stage, the plasma membrane was disrupted in a Dounce homogenizer without apparent damage to the microsoms, mitochondria, or nuclei. The homogenate was spun down (100 x g for 5 min), and the supernatant containing plasma membrane microsomes and intact mitochondria was separated from the pellet which contained the intact nuclei. The supernatant fraction was centrifuged (100,000 x g for 1 hr) to give the solubilized plasma membrane in the supernatant and the microsomal-mitochondrial fraction in the pellet. Although the microsomal fraction could be separated from the mitochondrial fraction by differential centrifugation, no attempt was made to further separate these 2 fractions. This method is readily reproducible with a yield approaching 80%. Criteria for the purity and homogeneity of each fraction were determined by enzymatic markers and specific labeling of each fraction. Table 1 shows the relative distribution of 2 enzymatic markers, the 5'-nucleotidase (a plasma membrane marker) and NADH oxidase (a microsomal marker), in the plasma membrane and in the microsomal-mitochondrial fractions, respectively. The incorporation of tritium into galactose of externally labeled glycoproteins in these 2 fractions is also shown in Table 1.

Analysis of the Proteins of the Various Fractions. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed in gradient slab gel (9 to 18% acrylamide) as described by Laemmli (19). Extracts from equal numbers of cells were diluted 1:1 with sample buffer, and 10 to 50 μg of protein were applied into each slot. The proteins on the gels were visualized by the Coomassie blue stain (R-250; Bio-Rad Laboratories, Rockville Centre, N. Y.), and the radioactive bands were identified by autoradiography of dried gel pages on sheets of Dupont Cronex safety film.

Reticulocytes and Erythrocytes. Reticulocytes were obtained from phenyldrazine-treated DBA/2J mice. The blood, which contained more than 80% reticulocytes, was collected from the eye. It was washed 3 times in 0.9% NaCl solution and fractionated as described for FL cells.

RESULTS

The Rate of Synthesis and Accumulation of Plasma Membrane Proteins. No appreciable difference in the rate of synthesis (Fig. 1) and accumulation (Fig. 2) of plasma membrane proteins, including spectrin, actin, and Band 3 (6), was found between FL cells incubated with HMBA (4 mM) and the untreated cells. In both, the bulk of the spectrin band was associated with the 100,000 x g supernatant of the cytosol (Figs. 1B and 2B), and only a small fraction with the plasma membrane protein bands comigrating with actin and Band 3 were distributed equally between the 2 fractions. Small amounts of both actin and spectrin were also found in the microsomal-mitochondrial fraction (Figs. 1C and 2C).
Membrane Protein. FL cells pulsed with H$_3^{32}$PO$_4$ for 3 hr to allow steady-state labeling of ATP (14) showed significant turnover of $^{32}$P in the various fractions of the cell (Chart 1a). In contrast, when the cells were incubated with HMBA (Chart 1b), there was a marked decrease in the amount of phosphate incorporated in the plasma membrane fraction. This was evident as early as 24 hr after the onset of induction. Analysis of the proteins of each fraction on slab gels showed a marked decrease of the phosphorylation level of the plasma membrane proteins (Fig. 3). The slight decrease in the level of phosphorylation of the protein fraction (100,000 × g) of the cytosol was evident in the supernatant (Fig. 4) and the pellet (results not shown). Actin was highly phosphorylated in all the fractions while spectrin was not. These biochemical changes were associated with differentiation, since cell line DR10, which is Me$_2$SO resistant (11), only showed decreases in the rate of phosphorylation of the plasma membrane proteins when incubated with HMBA to which it responds (50% benzidine-positive cells). In addition, when inhibitors of differentiation, such as dexamethasone (30) or TPA (29), were added to FL cells in combination with the inducer, phosphate incorporation and synthesis of proteins, including actin and spectrin, remained the same as in the untreated cells (results not shown).

In order to determine whether comparable changes occurred during normal erythropoiesis, the rate of phosphorylation of spectrin and actin in mouse erythrocytes and reticulocytes was studied. Fig. 5 shows the results of a typical experiment in which uninduced and induced FL cells were compared to reticulocytes and erythrocytes. Spectrin and actin from reticulocytes and mature erythrocytes were associated with the plasma membrane and the microsomal-mitochondrial fraction, and only trace amounts were detected in the cytosol. In contrast, spectrin from induced as well as from uninduced FL cells was detected mostly in the cytosol, and only a small amount was associated with the plasma membrane fraction. There were also other marked differences between mouse erythrocytes and reticulocytes and induced or uninduced FL cells. While the bulk of the proteins in the cytosol fraction of erythrocytes and reticulocytes was the $\alpha$- and $\beta$-globins, there were many other soluble proteins in the induced and uninduced FL cells. These proteins appear in minute amounts in reticulocytes and in trace amounts in mature erythrocytes. When the proteins from the various fractions of the cells pulse labeled with H$_3^{32}$PO$_4$ were analyzed on sodium dodecyl sulfate slab gel electrophoresis, substantial amounts of phosphate were incorporated into the reticulocyte spectrin (Band 2) and actin. Less but significant amounts were incorporated into the same proteins of mouse erythrocytes. On the other hand, although uninduced FL cells showed very high turnover of phosphate in the proteins, including the actin band, the spectrin band incorporated very low amounts of phosphate (autoradiogram not shown).

**DISCUSSION**

The rate of synthesis and accumulation of protein bands tentatively identified as spectrin, actin, and Band 3 (M.W. 100,000) in differentiated and undifferentiated FL cell clone DS19 were compared. Direct biochemical analysis of the cell fractions revealed no differences between the induced and the untreated cells, suggesting that FL cells originate from erythroid precursors which are capable of synthesizing these proteins at the time they are transformed by the virus. Our results, however, are at variance with those obtained by other investigators, possibly because different methods were used in their studies. Using the indirect double-antibody technique, Eisen et al. (5) observed a 15-fold increase in the amount of spectrin in induced cells as compared to only a 4-fold increase observed by Rossi et al. (28). Our data demonstrating that dexamethasone and TPA, compounds which inhibit differentiation, had no effect on the synthesis of all membrane proteins including spectrin, actin, and Band 3 in induced or uninduced cells are also at variance with the report of Fibach et al. (7) that TPA treatment caused a decrease in the amount of spectrin synthesized by HMBA-treated as well as by control cells. The method used by these investigators to measure spectrin levels was flow microfluorometry which measures the fluorescence:light scatter ratio of the cells and fluorescence intensity. Light scatter changes in a linear fashion with cell volume which is affected by the inducers (15, 21) as well as by TPA and reflects the effect of these agents on the cell cycle (12, 15). Thus, a source of error in interpreting the data may be introduced since there may be a change in the fluorescence:light scatter ratio because of the decrease in size of the differentiated cells, which has no bearing on the actual fluorescence intensity.

Another finding of interest revealed in the analysis of the cell fractions was that there were substantial amounts of protein corresponding to the typical membrane-associated RBC proteins, actin and particularly the spectrin in the 100,000 × g cytosol supernatant fraction, indicating that there may be cytoplasmic counterparts of membrane proteins (1). Only a small fraction of the spectrin was associated with the plasma membrane. A great part of the spectrin was recovered in the 100,000 × g pellet of the cytosol fraction which could be dissolved by treatment with 0.1 M dithiothreitol, suggesting that the bulk of it was cross-linked with $\mathrm{S-S}$ linkages to form a multimeric aggregate. Such aggregation of spectrin in pathological states was reported by Palek and Liu (25). FL cells are malignant although they can be induced to mimic functions of...
their normal counterparts. That spectrin from FL cells and mouse reticulocytes or erythrocytes differed from each other in several respects may be a reflection of the abnormal state of the cells. Spectrin from induced as well as untreated FL cells (M.W. 200,000 to 220,000 bands) did not comigrate with spectrin from reticulocytes (M.W. 200,000 to 240,000 bands) and incorporated very little labeled phosphate as compared to reticulocyte-mature erythrocyte spectrin. Since preliminary immune precipitation studies with mouse antispectrin antibodies indicate cross reactivity of the high-molecular-weight doublet with mouse erythrocyte spectrin, the differences observed might be due to a partial deletion of the polypeptide chains which might possess the phosphorylation moiety as well as the anchorage moiety in normal spectrin molecules. Experiments are underway to compare the fingerprints of FL spectrin to those of normal spectrins.

On the other hand, the M.W. 45,000 and 100,000 protein bands appeared in substantial amounts in the FL cell plasma membrane fraction, incorporated substantial amounts of labeled phosphate, and comigrated with the same proteins obtained from mouse erythrocytes. However, it should be noted that the FL cell M.W. 100,000 band (Band 3) differed from the erythrocyte Band 3 protein in that it appeared to be heterogeneous rather than homogenous (32). The possibility that Band 3 is not functioning is supported by the recent findings of Harper and Knauf (18). They reported that chloride transport, which is mediated by Band 3 in normal cells, showed very little increase in differentiated FL cells. Their results are also compatible with the findings of Sabban (7) who found no increase in the content of Band 3 in induced FL cells using the indirect double-antibody technique. In our study, the major RBC proteins have been identified mainly according to their migration properties compared to their normal RBC counterparts and thus all our conclusions await more positive identification by means of affinity labeling (Band 3) and immunoprecipitation (spectrin and actin). Isolation of these proteins and tryptic digest maps will add valuable information as to their nature.

Evidence that HMBA caused an early dephosphorylation of cellular proteins, particularly the plasma membrane proteins, was also obtained. Me₂SO and sodium butyrate caused a similar change (data not shown). This effect, which occurred early after the cells were exposed to the inducers, appears to coincide with the effect on cell transport (2, 11, 16) and paralleled the depletion of ATP (15). Our results are in agreement with those of Harel et al. (17), which showed a similar decrease in the rate of phosphate incorporation into the phospholipids of Me₂SO-treated FL cells of line 745. The changes in phosphate metabolism appear to be related to the differentiation process and perhaps reflect the alterations occurring on the plasma membrane of the treated cells.

This hypothesis is supported by the findings that dexamethasone, an inhibitor of induced differentiation, prevented the effect of HMBA on phosphate metabolism and that the Me₂SO-resistant cell line DR-10 showed a decrease in phosphate incorporation only when treated with HMBA but not when treated with Me₂SO.

REFERENCES

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Fig. 2. Polyacrylamide gel electrophoresis of the various fractions of differentiating FL cells. See legend to Fig. 1. Gels were stained for 30 min with Coomassie blue [0.25% (w/v) in 50% methanol:10% acetic acid] and destained for 48 to 72 hr with 10% acetic acid. Note the increase in the amount of globin in induced cells 2 to 3 days after onset of induction as compared to the amount of spectrin, actin, and Band 3 (100 KD).

Fig. 3. Effect of HMBA on the rate of 32P incorporation into the proteins of the plasma membrane of differentiating FL cells. Cell labeling and fractionation detailed in “Materials and Methods.” A, Coomassie blue stain; B, autoradiograph. See legend to Fig. 1. Note the molecular weight of the spectrin doublet is 200,000 to 220,000 and not 220,000 to 240,000 as in normal mouse erythroid cells. Lanes in B correspond to those in A.
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Fig. 4. Effect of HMBA on the rate of $^{32}$P incorporation into the proteins of the cytosol fraction (100,000 x g supernatant) of differentiating FL cells. See legend to Fig. 3. Lanes in B correspond to those in A.

Fig. 5. Polyacrylamide gel electrophoresis of the cytosol, microsomal-mitochondrial (ER), and plasma membrane fractions of FL cells as compared to mouse reticulocytes and mature erythrocytes. Lanes 1 and 7, mouse reticulocytes (R); Lanes 2, 5, and 9, mouse reticulocytes (R); Lanes 3 and 10, uninduced FL cells; Lanes 4, 6, and 10, induced FL cells. All cells were fractionated and processed as described in "Materials and Methods." The proportion of benzidine-positive FL cells in cultures treated for 3 days with 4 mg HMBA was 79%. Numbers on right, molecular weights of the markers described in the legend to Fig. 1.
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