Interferon-independent activation of (2'-5') oligoadenylate synthetase in Friend erythroleukemia cell variants exposed to HMBA

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SUMMARY

To provide evidence for the implication of interferon (IFN)-induced proteins in the regulation of cell growth during differentiation, the activation of (2'-5') oligoadenylate synthetase (2-5A synthetase) as well as of PKR, two IFN-induced proteins, during differentiation of Friend erythroleukemia cells, was studied. Two cell variants were used. The first (FL) was completely susceptible to hexamethylene bis-acetamide (HMBA)-treatment and responded in both growth-retardation and hemoglobin synthesis. The second (R1) failed to synthesize hemoglobin in response to HMBA although cell growth was still inhibited. In both cell variants, 2-5A synthetase enzyme activity was induced in a similar fashion, reaching a peak at 26 hours after treatment with HMBA. However, the down regulation of activity thereafter was not identical in both cases. In R1 cells, the reduction was much slower compared to FL cells. A similar pattern was observed with the appearance of the 43 kDa isoform of 2-5A synthetase in immunoblots. An analysis of 2-5A synthetase gene expression revealed the presence of 1.7 kb transcripts which peaked at 16 hours after HMBA-treatment in both cell variants. Again, the down-regulation in expression was slower in R1 than in FL cells. Addition of anti-murine α/β-IFN antibodies did not reduce the level of either 2-5A synthetase expression or enzyme activity in either cell variant. Interestingly, the presence of antibodies also did not affect the pattern of pRb phosphorylation in the cell variants exposed to HMBA. In both cell variants, an increase in the amount of the phosphorylated form (ppRb) was observed in immunoblots after 4 hours. This form was gradually transformed to the underphosphorylated molecule (pRb) with time in culture, even in the presence of antibodies. This further substantiates the notion that IFN-induced regulation of pRb phosphorylation is mediated by IFN-induced proteins.

The basal level of either expression or enzymatic activity of PKR detected in untreated FL or R1 cells, was relatively high. Treatment with HMBA did not result in further induction of PKR in either cell variant.

Key words: Interferon, 2-5A synthetase, Erythroleukemia cell

INTRODUCTION

Friend erythroleukemia (FL) cells are erythroid cell precursors that are virus-transformed and resemble the erythroid colony forming unit (Friend et al., 1971). Upon treatment with a variety of inducers, including dimethyl sulfoxide (DMSO) and hexamethylene bis-acetamide (HMBA) (Friend et al., 1971; Reuben et al., 1976), the cells undergo terminal differentiation accompanied by the accumulation of hemoglobin (Fibach et al., 1977). In addition, the reduced expression of a number of proto-oncogenes during the differentiation of FL cells has been observed (Giroldi et al., 1988). The down-regulation of c-myc expression in differentiating cells, for example, has been shown to occur both at the p1 and p2 promoters of this gene (Kohlhuber et al., 1993). Other reports indicate that the regulation of expression of either c-myc or c-myb is a consequence of premature transcription termination and increased turnover of mRNA and protein molecules (Spotts and Hann, 1990; Watson, 1988). In all these cases, the physiological agents that are directly responsible for down-regulation of proto-oncogene expression and for cell-growth arrest of differentiating FL cells are still unknown.

It is now well accepted that interferon (IFN)-induced proteins play a role in the regulation of cell growth (Romeo et al., 1992). A direct proof for this notion was provided by a series of transfection studies. Thus, a human glioblastoma clone harboring a cDNA encoding the small molecular mass (2'-5') oligoadenylate synthetase (2-5A synthetase), a well known IFN-induced protein, showed a reduced rate of cell proliferation (Rysiecki et al., 1989). In another report, it has been demonstrated that transfection of a dominant negative mutant of RNase L, an enzyme activated by 2-5A oligomers, the enzyme product of 2-5A synthetase, resulted in a reduced rate of cell proliferation (Rysiecki et al., 1989). In another report, it has been demonstrated that transfection of a dominant negative mutant of RNase L, an enzyme activated by 2-5A oligomers, the enzyme product of 2-5A synthetase, resulted in the suppression of the IFN-mediated antiproliferative effect (Hassel et al., 1993). Similarly, expression of a negative-dominant mutant of PKR, another IFN-induced protein, resulted in malignant transformation of NIH/3T3 mouse fibroblasts, most likely due to inhibition of the endogenous gene activity (Koromilus et al., 1992). Taken together, these reports confirm the significance of IFN-induced proteins during cell growth.
A functional role for the IFN-system in cell-growth regulation was demonstrated by the finding that IFN and other cytokines suppress the phosphorylation of the retinoblastoma protein (pRb) in hematopoietic cells (Resnitzky et al., 1992). pRb in its underphosphorylated form binds to the transcription factor E2F. This factor has been shown to be involved in cell replication (Lees et al., 1993). A link was therefore established between the growth-suppressor activity of IFN and a biochemical event which leads to cell-growth arrest. It is still not clear, however, whether IFN-induced proteins play a role during differentiation in an IFN-independent manner. The induction of 2-5A synthetase activity as a result of exposure to growth factors rather than to IFN has been demonstrated (Garcia-Blanco et al., 1989; Lin et al., 1983; Saarma et al., 1986). In addition, incubation of primary myoblasts in the presence of anti-α/β-IFN antibodies did not reduce the level of 2-5A synthetase activity induced during myogenesis (Birnbaum et al., 1983). It is, therefore, possible that a set of factors different from those activated by IFN might be responsible for the induction of this and other IFN-induced proteins.

In this study, we show that 2-5A synthetase expression and activity are induced in two different variants of FL cells in response to treatment with HMBA, even in the presence of anti-α/β-IFN antibodies. Furthermore, the alternation in pRb phosphorylation observed during differentiation of FL cells is also α/β-IFN-independent, suggesting that these cytokines are not involved in the differentiation process. In contrast, PKR expression and activity are not enhanced above the basal level in the same variants exposed to HMBA.

MATERIALS AND METHODS

Cell cultures

Two variants of Friend erythroleukemia cells were used throughout the study. FL cells represent the normal phenotype and the cells are susceptible to differentiation by both HMBA and DMSO. In contrast, R1 cells are resistant and do not synthesize hemoglobin in response to either agent (Marks et al., 1983). Both variants were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Biological Industries, Beth Haemek, Israel) and Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Biological Industries, Beth Haemek, Israel) and were transferred twice weekly. For experimental purposes, cultures were prepared with 2×10⁶ cells/ml in 10 cm tissue culture plates. After overnight incubation, N,N'-hexamethylene bis-acetamide (HMBA, Sigma Chemical Co., St Louis, MO) was added at a final concentration of 5 mM. Mouse α/β-IFN (Lee Biomolecular, San Diego, CA) was added to untreated culture at a concentration of 50 international units (i.u.)/ml and anti mouse α/β-IFN antibody (Lee Biomolecules) was added at 200 i.u./ml. The cells were collected at the indicated times, centrifuged at 800 g for 5 minutes, washed once with phosphate buffered saline (PBS) and subjected to analysis as indicated for each experiment. For growth curves, cells in the plate were gently resuspended with a pipette. 0.5 ml samples were withdrawn and mixed with an equal volume of 0.4% trypan blue in normal saline (Gibco, Grand Island, NY). The number of unstained cells was determined in a hemocytometer.

Hemoglobin determination

The percentage of cells synthesizing hemoglobin (stained in blue) was determined by the benzidine staining method (Orkin et al., 1976). Briefly, a benzidine solution was prepared by adding 20 μl of 30% hydrogen peroxide to 1 ml of 0.2% benzidine in 0.5 M acetic acid. About 1 ml of cell suspension was centrifuged at 800 g for 5 minutes. The cells were resuspended in 100 μl of PBS and mixed with 50 μl of fresh benzidine solution. Approximately 200 cells were counted.

Preparation of cell extracts (S-10)

Cells were harvested, sedimented, washed once with PBS, and sedimented again. Extracts were prepared by adding ice-cold lysis buffer containing 20 mM Heps, pH 7.5, 5 mM Mg-acetate, 0.5% NP-40, 1 mM dithiothreitol, 10% glycerol and 1 mM EDTA. After 5 minutes in ice, the extracts were centrifuged at 1,000 g for 10 minutes, and the supernatant centrifuged at 10,000 g for 10 minutes. The soluble fractions (S-10) were stored at −70°C until use.

Determination of 2-5A synthetase and PKR activities

The 2-5A synthetase activity in cell extracts (S-10) was determined as previously described (Gopas et al., 1992) using 20 μg protein and poly(I) poly(C)-agarose beads (Pharmacia, Uppsala, Sweden). The 32P-labelled 2-5A oligomers formed were treated with 50 units/ml of calf intestine alkaline phosphatase (Boehringer, Mannheim, Germany) for 2 hours at 37°C and samples containing 5 μl each were placed on polyethyleneimine-cellulose chromatography sheets as described (Gopas et al., 1992).

For the determination of PKR, heparin (50-100 units/ml) was added to S-10 samples containing 500 μg of protein. The samples were then incubated at 4°C for 10 minutes. An equal volume of poly(I)poly(C)-agarose beads was added at room temperature for 30 minutes with occasional gentle mixing. The beads were washed several times with buffer B containing 50 mM KCl, 2 mM Mg-acetate, 7 mM β-mercaptoethanol, 20% glycerol, 10 mM Hepes, pH 7.6, and then once in buffer C (buffer B with 5 mM MnCl2).

The final pellet was resuspended in buffer C supplemented with 1 μCi [γ-32P]ATP (50-100 Ci/mmol; Amersham) and incubated for 30 minutes at 30°C. After centrifugation, the pellet was washed three times with buffer C and resuspended in 1 vol. buffer C and 1 vol. electrophoresis sample buffer containing 6% SDS (w/v), 30% glycerol (w/v), 0.02% Bromophenol blue (w/v), 200 mM Tris-HCl, pH 6.8, and 250 mM β-mercaptoethanol. Samples were then heated at 96°C for 5 minutes and centrifuged. The supernatants were collected and analyzed on 10% polyacrylamide slab gels containing SDS. The gel was dried and the phosphorylated proteins were detected by autoradiography on Fuji RX film.

Identification of proteins by immunoblotting

The identification of 2-5A synthetase or pRb proteins in cell extracts (S-10) was performed by western blotting analysis. Samples containing 40 μg of protein were suspended in 30 μl of 0.5 M Tris-HCl, pH 6.8, 10% glycerol, 10% SDS, 0.05% β-mercaptoethanol and 0.05% Bromophenol blue, boiled for 3 minutes and loaded on a 10% polyacrylamide-SDS gel. As molecular mass markers, we used the rainbow colored protein molecular mass markers supplied by Amersham International Plc (Amersham, UK). Electrophoresis was in the cold at 50 V for 15 minutes and 200 V for another hour. Transfer to nitrocellulose sheets was performed in the miniTrans-blot cell (Bio-Rad Laboratories, Hercules, CA) at 4°C in a buffer containing 25 mM Tris-HCl, pH 8.3, 192 mM glycine and 20% methanol for 1 hour at 200 mA. The nitrocellulose sheet was immersed in blocking solution containing 2% bovine serum albumin (BSA), 0.02% Tween-20 in PBS for 18 hours at 4°C. It was then transferred to a blocking solution supplemented with either a 1:500 dilution of rabbit polyclonal antibodies directed against fragment B common to all isoforms of 2-5A synthetase (Chebath et al., 1987) or with a 1:500 dilution of mouse anti-human retinoblastoma monoclonal antibodies (Cat. no. 14100A; PharMingen, San Diego, CA), incubated for 2 hours at 37°C and then washed 3 times with 0.02% Tween-20 in PBS. As a secondary detection antibody, we used peroxidase labelled anti-rabbit or anti-mouse antibodies, and detection was performed by the ECL Western blotting procedure as described by the supplier (Amersham). Light emission was detected by a 2 minute exposure to Fuji RX Medical X-ray film.
Activation of 2-5A synthetase in FL cells

RNA preparation and hybridization

Total RNA from rat skeletal muscle cultures harvested at the indicated times after seeding, was purified by LiCl-urea precipitation and phenol-chloroform extraction as previously described (Auffray and Rougeon, 1980). In one set of experiments (repeated with 3 different sets), we used the same RNA preparations for the analysis of all types of transcripts described in this study. For the preparation of northern blots, samples containing 20 µg RNA were analyzed on a 0.9% agarose gel in running buffer containing formaldehyde followed by blotting onto nitrocellulose membrane filters as described (Sambrook et al., 1989).

The amount of RNA loaded in each lane was assessed by ethidium bromide staining and the profile is shown in the appropriate figures.

For hybridization, the nitrocellulose filters were first prehybridized overnight at 65°C in a solution containing 0.5 M Na2HPO4, pH 7.2, 1% BSA crystalline (w/v), 1 mM EDTA, pH 8.0, and 7% (w/v) SDS. Hybridization was performed at 65°C for 24 hours in the same buffer containing 1-2×10^6 cpm/ml of 32P-labelled probe. At the end of the hybridization period, blots were washed twice with 1× SSPE and 0.1% SDS at 50°C for 30 minutes each and twice with 0.1× SSPE and 0.1% SDS at 50°C for 30 minutes each. For hybridization of the same blots, membrane filters were soaked in H2O and boiled for 2 minutes, followed by two washings with H2O at room temperature. Filters were then exposed for 10 days for autoradiography, to ensure the removal of radioactivity.

Scanning of radioactive bands was performed by the GS300 Densitometer (Hoefer Scientific Instruments, San Francisco, CA).

Probes

The following probes were used in this study: (1) a mouse cDNA clone (E-MoL3) isolated by J. Chebath and E. M. Coccia from the Virology Department, Weizmann Institute of Science, Rehovot, Israel. The L3 insert is a 1.3 kb EcoRI fragment encoding active mouse 2-5A synthetase enzyme (43 kDa isoform) and representing a copy of the 1.7 kb transcript (David et al., 1989). (2) A 2 kb fragment excised by BamHI and PstI from the plasmid pGEM3Z. This fragment contains the entire coding sequence for murine PKR (Feng et al., 1992).

All probes were labelled with [32P]dCTP (specific activity 3,000 Ci/m mole, Amersham) using the rapid multiprime DNA labelling kit as recommended by the suppliers (RAN.1601; Amersham). The specific activity was 2-8×10^6 cpm/µg.

RESULTS

Replication and differentiation of FL and R1 cells

Since we wanted to compare the inducibility of 2-5A synthetase or PKR in two Friend erythroleukemia cell variants which differ in their response to HMBA, we first established the growth-rates of FL and R1 cells in the presence and absence of this differentiating agent. The cells were subcultured at 2×10^5 cells/ml into two separate groups of 10 cm tissue culture plates and after an overnight incubation, 5 mM HMBA was added to one group. The other served as a control. The cell number was then determined at different times thereafter in 3 different plates at each point. The results presented in Fig. 1A show that the growth-rate of R1 cells was higher than that of FL cells. HMBA reduced the growth rate of both cell lines. However, its effect on R1 cells was somewhat smaller than that observed with FL cells. The percentage of benzidine-positive cells, a measurement of hemoglobin synthesizing cells was then determined in the same plates of both cell lines. It is clearly shown (Fig. 1B) that while the percentage of benzidine-positive FL cells increased significantly with time after HMBA treatment, reaching 90% at 72 hours after treatment, only 10% of R1 cells became benzidine-positive at the same time, indicating that R1 cells are not responding in hemoglobin synthesis to HMBA-treatment.

Effect of HMBA on expression, appearance and enzyme activity of 2-5A synthetase in FL and R1 cells

To establish whether 2-5A synthetase is activated during differentiation of FL or R1 cells in response to HMBA-treatment, we determined the kinetics of 2-5A synthetase activity and the appearance of 2-5A synthetase protein at different times after treatment. Extracts were prepared and analyzed for either the enzyme activity by using TLC or for the level of 2-5A synthetase by using western blot analysis. As shown in Fig. 2A, 2-5A synthetase activity in untreated FL cells increased only slightly with time in culture, with less than a 2-fold increase by 72 hours. In HMBA-treated cultures, on the other hand, a peak activity was observed at 48 hours after treatment followed by a decrease thereafter. The results obtained with R1 cells (Fig. 2B) indicate that similar to FL cells, a small increase in activity with time of incubation was observed in untreated cells. In HMBA-treated cultures, an increase in activity by 4 hours was already evident followed by a further increase by 28 hours. However, the reduction in activity thereafter was much slower, indicating that the kinetics

Fig. 1. Rates of cell growth and hemoglobin synthesis in FL and R1 cells exposed to HMBA. Cultures were prepared and treated with HMBA one day later. At daily intervals, samples were withdrawn from 3 plates at each point, and the number of live cells was determined by trypan blue exclusion assay (A). In parallel samples, the number of hemoglobin synthesizing cells was determined by benzidine staining (B). The standard deviation is indicated by the bars.
of 2-5A synthetase activity in FL and R1 cells are not completely identical. These results are supported by the data on the presence of 2-5A synthetase protein in cell extracts (Fig. 2C). Western blot analysis using antibodies directed against fragment B common to all forms of human 2-5A synthetase (Chebath et al., 1987) revealed a major species of 43 kDa. Other isoforms were not visible under the same conditions. In FL cells, the highest amount of protein was observed at 24 hours. On the other hand, in R1 cells, an increase in the amount of protein compared to untreated cells (collected at 24 hours after the initiation of the experiment) was evident 4 hours after treatment. A slight increase was then observed at 24 hours with no significant changes at 48 or 72 hours. Although not absolutely identical, these results resemble those obtained with the kinetics of enzyme activity. It should be noted that when cell extracts prepared from FL cells treated for 24 hours with α/β-IFN were similarly analyzed, the three forms of 2-5A synthetase of 43, 70 and 101 kDa present in mouse cells were visible (IFN in Fig. 2D). This is in contrast to the single 43 kDa isoform observed in cells treated for 24 hours with HMBA (HMBA in Fig. 2D). In untreated FL cells (c in Fig. 2D), mostly non-specific bands were detected with a minor band of the 43 kDa isoform.

We then proceeded to determine the level of 2-5A synthetase-specific RNA transcripts in HMBA-treated FL or R1 cells. Total RNA was extracted at different times after treatment or from untreated cells collected at 16-24 hours after the initiation of the experiment. The RNA was analyzed by northern blotting using as a probe the L3 cDNA coding for the 43 kDa form of murine 2-5A synthetase (David et al., 1989). The results (Fig. 3A) show that a significant increase in the level of transcripts in both FL and R1 cells was visible only at 16 hours after treatment (lanes 3 and 11) followed by a reduction thereafter. Interestingly, as observed with the enzyme activity (Fig. 2A and B), the rate of reduction in R1 cells was slower than that seen with FL cells (Fig. 3C). It appears that the rate of appearance of RNA transcripts precedes by few hours that of 2-5A synthetase activity.

It should be noted that in parallel to an increased growth rate of R1 cells, a higher level of both enzyme activity (Fig. 2A and B), and gene expression (Fig. 3) was observed in these cells in comparison to FL cells. However, this was not the case with the appearance of 2-5A synthetase protein (Fig. 2C). We think that this reflects fluctuations in experimental conditions.

Induction of 2-5A synthetase expression and activity by HMBA in the presence of anti-α/β-IFN antibodies

The activation of 2-5A synthetase activity in FL subclones by
HMBA could be a result of IFN-release by these cells during differentiation. To clarify this point, FL cultures were treated with HMBA in the absence and presence of 200 U/ml of anti-mouse α/β-IFN antibodies. As a positive control we used cultures treated either with 50 IU/ml of mouse α/β-IFN or with the same dose of IFN and 200 U/ml of anti-α/β-IFN antibodies. All cells were collected at 28 hours after the addition of HMBA, cell extracts were prepared and analyzed for 2-5A synthetase activity. As illustrated in Fig. 4, treatment with HMBA (H) increased the activity over control (Ct) values by 5-fold. Addition of anti-α/β-IFN antibodies (Ab) did not alter the level of enzyme activity in HMBA-treated cultures (H+Ab). On the other hand, the addition of antibodies to IFN-treated cultures (IFN+Ab), reduced the level of 2-5A synthetase activity to control values, although treatment with IFN alone resulted in extremely high enzyme activity (IFN). Addition of antibodies only to cultures did not affect the level of 2-5A synthetase activity (Ab). We conclude that murine α/β-IFN is most likely not involved in the induction of 2-5A synthetase activity in our cell system.

To further prove this notion, we determined the level of 2-5A synthetase-specific RNA transcripts in both FL and R1 cultures treated with HMBA in the absence and presence of antibodies. The results indicate that HMBA treatment, as expected, caused an increase in the amount of specific RNA transcripts with a peak at 16 hours after treatment, both in FL and R1 cells (Fig. 5A,C). The presence of anti-α/β-IFN antibodies did not reduce the level of transcripts (lanes 5-7 and 14-16 in Fig. 5A), and in the case of R1 cells, an even higher level of transcripts was observed (compare lanes 11-13 to lanes 14-16), for which we have no explanation. In contrast, the presence of antibodies in IFN-treated cultures reduced dramatically the level of transcripts as compared to cultures treated with IFN alone (Fig. 5A, lanes 8, 9, 17, 18).

**Effect of HMBA on expression and activity of PKR in FL and R1 cells**

Since PKR is another known IFN-induced protein which has been recently implicated in the regulation of cell growth (Koromilas et al., 1992), we determined its expression and enzyme activity during differentiation of FL and R1 cells. Both cultures were treated with HMBA and at daily intervals PKR activity was determined in cell extracts by establishing the level of phosphorylated P65 which is the murine enzyme molecule autophosphorylated during the enzyme reaction. It seems that the level of PKR activity in untreated cultures of both FL and R1 origin is somewhat higher than that detected in HMBA-treated cells (Fig. 6). However, we could not detect a significant difference in enzyme activity between 4 and 72 hours after treatment. These results were further supported by analysis of total RNA extracted at different times after treatment with HMBA for PKR-specific transcripts. The results shown in Fig. 7 indicate that the same level of transcripts appeared at all time points, including in untreated cultures. We conclude that PKR is expressed even in untreated FL variants and that the level of expression is not significantly altered during differentiation.

**Alterations in the phosphorylated state of pRB during differentiation of FL and R1 cells**

pRb is posttranslationally modified during the cell cycle, being hypophosphorylated at G0 and G1 and then undergoing additional phosphorylation as the cells enter late G1, S, G2 and most of the M phase (Hinds and Weinberg, 1994). Moreover, IFN has been shown to modify the phosphorylated state of pRb (Resnitzky et al., 1992). We, therefore, reasoned that the
Fig. 5. Expression of 2-5A synthetase in FL or R1 cells exposed to HMBA and anti-mouse α/β-IFN antibodies. Total RNA was extracted from FL or R1 cells at the indicated times after treatment with HMBA or simultaneously with HMBA and anti-α/β-IFN antibodies. Samples containing 20 µg of RNA were subjected to northern blot analysis and probed with 32P-labelled L3 cDNA.

(A) Autoradiogram of the blot containing the following samples: lanes 1 and 10, untreated cells collected at 0 time (one day after seeding); lanes 2-4 and 11-13, cells collected at 8, 16 and 24 hours after treatment with HMBA, respectively; lanes 5-7 and 14-16, cells collected at 8, 16 and 24 hours after treatment with HMBA and antibodies, respectively; lanes 8 and 17, cells treated with mouse α/β-IFN only for 24 hours; lanes 9 and 18, cells treated with IFN and antibodies. (B) Ethidium bromide staining profile of the gel. (C) Densitometric scans of the bands shown in A. The percentage of maximal intensity in each section (FL or R1) is presented. Hatched columns, cells treated with HMBA. Open columns, cells treated with HMBA and antibodies.

Fig. 6. PKR activity in FL and R1 cells exposed to HMBA. Cell extracts were prepared at the indicated times (hours) after HMBA-treatment and analyzed for PKR activity. (A) Autoradiogram of the gel showing the autophosphorylated PKR molecule (p65). (B) Scanning profile of the bands shown in A. (C) Untreated cells collected at 24 hours.
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Activation of 2-5A synthetase, an IFN-induced protein, during differentiation of FL cells, may correlate to changes in pRb phosphorylation. Both FL and R1 cells were treated with HMBA (or remained untreated). Part of the cultures received, in addition, anti-α/β-IFN antibodies. Cell extracts were prepared and analyzed for the presence of pRb by western blotting using a monoclonal antibody which detects both the phosphorylated and non-phosphorylated proteins. The results show that the amount of either form of pRb is very low, both in FL and R1 cells (Fig. 8). However, a rapid increase in the amount of the phosphorylated form detected at 4 hours after HMBA-treatment was evident. This amount gradually decreased with a concomitant increase of the hypo-phosphorylated form with time in culture. The same pattern was observed with both cell lines. Addition of anti-α/β-IFN antibodies to HMBA-treated cultures did not affect the level or kinetics of the appearance of pRb proteins during differentiation of the FL variants, indicating that release of IFN is not directly involved in this process as seen earlier with 2-5A synthetase expression and enzyme activity.

DISCUSSION

It is well established now that most biological activities attributed to IFN are mediated by IFN-induced proteins (Romeo et al., 1992). It is tempting to postulate, therefore, that when these proteins are activated during a physiological process, it usually will be a result of release of an endogenous type of IFN which functions in an autocrine fashion to induce the appropriate proteins. Indeed, in several hematopoietic systems, the release of β-type IFN during cell differentiation has been described (Resnitzky et al., 1986). Furthermore, in early reports, the release of endogenous IFN from FL cells has also been demonstrated (Kimchi, 1981). However, in a series of other reports, it has been shown that 2-5A synthetase activity is induced in an IFN-independent manner in some cell systems (Birnbaum et al., 1983; Garcia-Blanco et al., 1989; Lin et al., 1983; Saarma et al., 1986). Even in the case of HL-60 promyelocytic cells where the presence of anti-α-IFN antibodies has been shown to reduce the level of 2-5A synthetase expression and activity induced by dimethylsulfoxide (DMSO), no expression...
of the α or β-IFN genes or induction of the IFN-inducible HLA-B protein could be detected under the same conditions (Schwartz and Nilson, 1989). It is, therefore, still questionable whether IFN is directly involved in cell growth regulation and differentiation.

We used in our study two subclones of Friend erythroleukemic cells, one (FL) with wild-type properties being sensitive to differentiation by DMSO or HMBA, the other (R1) resistant to both agents. Our results demonstrate that in both cell variants, 2-5A synthetase expression and activity are induced in response to treatment with HMBA. The major difference between R1 and FL cells in the kinetics of these responses was the slower rate of reduction in all biological parameters used to characterize 2-5A synthetase in the resistant variant (Figs 2 and 3). As expected, R1 cells failed to synthesize hemoglobin following treatment with HMBA since they are deficient in their ability to undergo terminal differentiation (Marks et al., 1983). However, our results indicate that these cells are not deficient in their early responses to the differentiating agent. First, the growth rate of both FL and R1 cells were similarly affected by HMBA (Fig. 1A). Second, the Rb protein was induced by HMBA in a similar manner in both cell variants. Furthermore, the alteration in the phosphorylated state of pRb with time in culture was identical in these cells (Fig. 8). It should be mentioned in this context that the underphosphorylated form of pRb possesses the growth suppression activity, apparently by forming a complex with the E2F family of transcription factors (Lees et al., 1993), thus preventing the cells from entering the S phase (Hinds and Weinberg, 1994). Third, in agreement with previous reports (Kohlhuber et al., 1993; Spotts and Hann, 1990; Watson, 1988), we observed a similar reduction in expression of c-myc in FL and R1 cells (unpublished data). Finally, in both cell variants, the induction of 2-5A synthetase proceeded in a similar fashion in response to HMBA, indicating that this process is part of the early events triggered during differentiation of Friend erythroleukemia cells. Since the 2-5A system is responsible for the breakdown of mRNA molecules (Wreschner et al., 1981), it is reasonable to assume that the activation of this system early during differentiation is responsible for the down-regulation of genes associated with cell growth, a common feature for both FL and R1 cells. It should be mentioned in this context that earlier reports demonstrated the reduction in the level of cyclin A, coupled with a prolonged G1 and accumulation of underphosphorylated pRb in mouse erythroleukemia cells exposed to HMBA (Kiyokawa et al., 1993; Richon et al., 1992). However, no attempt to correlate this phenomenon to IFN-induced proteins was performed.

The results presented in this report show that type I interferons (α- or β-IFN) are, most likely, not involved in the response of the two cell variants to HMBA. This notion is based on the fact that antibodies directed against α/β-IFN did not interfere with expression of 2-5A synthetase (Fig. 5), its enzyme activity (Fig. 4) and phosphorylated state of pRb (Fig. 8) in either FL or R1 cells exposed to HMBA. This is in contrast to an early report which attempted to characterize a spontaneous interferon secreted during differentiation of FL cells (Friedman-Einat et al., 1982). We assume that a different subclone of Friend erythroleukemic cells was used in this study. However, the possibility that a specific sub-type of IFN secreted by FL cells and not neutralized by the antiserum used in this study cannot be ruled out. In a series of other reports, few FL subclones resistant to IFN were isolated (Affarbis et al., 1981, 1983; Coccia et al., 1988). Although these cells contain receptors to α/β-IFN, treatment with these cytokines did not induce 2-5A synthetase or PKR activities (Affarbis et al., 1983; Coccia et al., 1988), indicating that the cells lack some of the DNA-binding proteins which interact with the interferon response enhancer (Coccia et al., 1991). Nevertheless, DMSO induced differentiation to the same level in both IFN-sensitive and resistant variants. Moreover, addition of α/β-IFN did not enhance the differentiation of the resistant cells (Affarbis et al., 1981). These results indicate that the IFN-associated signal transduction machinery is not involved in the differentiation of FL cells. Hence, the differentiation cannot be mediated by a secreted type of β-IFN.

The factors that transduce the signal from the α/β or γ-IFN receptor to the cell nucleus have been recently characterized (Darnell et al., 1994). Receptor-bound Jak family of tyrosine kinases (Jak 1, Jak 2 and tyk 2) are activated following IFN binding. The target proteins phosphorylated by these kinases consist of a group of transcription factors - the STAT proteins. These proteins are translocated to the nucleus to form a complex that binds to an IFN-stimulated response element (ISRE) present in the promoter region of IFN-responsive genes (Pellegrini and Scindler, 1993). It became apparent now that the Jak-STAT interaction is a common feature for signaling by other cytokines as well and new members of both Jak and STAT proteins have been isolated (Asao et al., 1994; Kawamura et al., 1994; Rane and Reddy, 1994; Yamamoto et al., 1994; Stahl et al., 1995). It has been demonstrated that prolactin and interleukin 2 activate the interferon responsive factor 1 (IRF-1) by specific transcription factors different from those increased in IFN-signaling (Gilmour and Reich, 1994). Indeed, this factor as well as IRF-2 were recently shown to bind to ISRE in γ-IFN treated FL cells (Coccia et al., 1995). Finally, a recent report indicates that a unique factor binds to ISRE in response to viral infection or exposure to double-stranded RNA (Daly and Reich, 1994). Based on these and our results, we suggest that in response to HMBA-treatment of Friend erythroleukemia cells, differentiation-specific transcription factors are activated. These factors may be different from those participating in IFN-signaling.

In our study, we could not detect an enhancement of PKR expression and activity following exposure of FL or R1 cells to HMBA. However, a relatively high basal level of expression in undifferentiated cells was observed in this case (Figs 6, 7). The gene coding for mouse PKR was recently cloned and sequenced (Tanaka and Samuel, 1994). Although the promoter region contains an ISRE and its flanking sequences, they are not absolutely identical to the sequences present in the promoter region of 2-5A synthetase (Benech et al., 1987). It can therefore be argued that a different balance between negative (like ISGF-2; Kessler et al., 1989) and positive factors is needed to activate either PKR or 2-5A synthetase. An alternative explanation would be that PKR does not play any role in the differentiation of FL cells. A better understanding of the function that these and additional IFN-induced proteins fulfill during differentiation will be provided by transfection of FL and other cell types with expression vectors harboring the genes encoding these proteins followed by studies on the properties of the transfected cells.
REFERENCES


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