Activation of the pp60-src kinase during differentiation of monomyelocytic cells in vitro

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The proto-oncogene c-src, the cellular homolog of the Rous sarcoma virus (RSV) transforming gene v-src, is expressed in a tissue-specific and age-dependent manner. Its physiological function, although still unknown, appears to be more closely related to differentiation processes than to proliferation processes. To obtain more information about the physiological role of the c-src gene in cells, we have studied differentiation-dependent alterations using the human HL-60 leukemia cell line as a model system. Induction of monocyteic and granulocytic differentiation of HL-60 cells by 12-O-tetradecanoylphorbol-13-acetate (TPA) and dimethylsulfoxide (DMSO) is associated with an activation of the pp60-src tyrosine kinase, but not with increased c-src gene expression. Control experiments exclude an interaction of TPA and DMSO themselves with the pp60-src kinase.

Key words: c-src/differentiation/protein tyrosine kinase/proto-oncogene

Introduction

Cellular homologs of the retroviral transforming genes have been reported to play an important role during proliferation and differentiation processes (Gessler and Barnekow, 1984; Müller et al., 1984; Rüther et al., 1985; Genda and Metcalf, 1985; Mitchell et al., 1985; Sariban et al., 1985). These genes are highly conserved during evolution implying that they display essential functions under physiological conditions (Shilo and Weinberg, 1981; Schartl and Barnekow, 1982; Barnekow and Schartl, 1984). We have recently studied the expression of the proto-oncogene c-src, the cellular counterpart of the Rous sarcoma virus (RSV) transforming gene v-src, during embryonic development of three vertebrate classes and we have found that the expression of the c-src gene product, which is highly homologous to the transforming protein of RSV, pp60src (Takeya and Hanafusa, 1983), seems to be more closely related to differentiation processes than to proliferation processes. Expression is only barely detectable during the highly proliferative stages of early embryogenesis, whereas with the onset of organogenesis, all three classes show a drastic increase in expression (Schartl and Barnekow, 1984). These findings are consistent with data recently reported by Brugge et al. (1985). These authors report on a high level of expression of c-src kinase activity in post-mitotic neurones, which indicates that the activation of c-src expression does not correlate with cell proliferation.

In the present study we followed the expression of the c-src proto-oncogene by measuring its specific protein tyrosine kinase and the amount of c-src mRNA during in vitro differentiation of the human HL-60 leukemia cell line. HL-60 cells, isolated from the peripheral blood of a patient with acute promyelocytic leukemia (Collins et al., 1977), proliferate continuously in suspension culture and represent predominantly promyelocytes. These cells are induced to differentiate to cells having many morphological and functional changes by using a variety of compounds including dimethylsulfoxide (DMSO) or the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Addition of DMSO to HL-60 cells induces differentiation and leads to granulocyte-like cells, whereas in the presence of TPA the HL-60 cells differentiate into macrophages within a few days (Collins et al., 1978, Rovera et al., 1979).

Results

Determination of the pp60-src kinase in TPA treated HL-60 cells revealed a differentiation-dependent increase in the phosphorylating activity (Figure 1). A total of three different tumor-bearing rabbit (TBR) sera were used throughout the experiments, all with the same result. To confirm that it is indeed the heavy chain of the pp60-src antibody which is phosphorylated in the in vitro kinase assay, aliquots of each sample were run under non-reducing conditions and the radioactivity was then detected in the 150 kd IgG band (data not shown). Addition of sodium orthovanadate, known to be a potent phosphotyrosine-phosphatase inhibitor, to the extraction buffer, reduced the kinase activity precipitated from TPA treated and untreated cells to nearly undetectable levels (data not shown). Similar results have recently been reported for pp60-src kinase activity from normal cells (Courtine, 1985).

HL-60 cells, which in the presence of DMSO differentiate into granulocytes, displayed a differentiation-dependent increase in the expression of pp60-src kinase activity compared to untreated sister cultures (Figure 1).

In order to establish that the IgG heavy-chain phosphorylation was due to a tyrosine-specific kinase activity, we performed two-dimensional phosphoamino acid analysis. The 53 kd product of the in vitro protein kinase reaction seen in Figure 1 was cut out of the gel, eluted and the phosphoamino acids were analyzed. One example is shown in Figure 2. The phosphorylated IgG heavy chain is exclusively labeled in phosphotyrosine. For further characterization we tested the kinase activity in the presence of diadenosinetetraphosphate (Ap4A), a compound which exhibits an inhibitory effect on the viral pp60src kinase activity, but in concentrations up to 100 μM, does not seem to affect the cellular enzyme (Barnekow, 1983). In all cases investigated, the kinase activity was insensitive to inhibition towards Ap4A in concentrations from 1–100 μM (data not shown).

Quantitative determination of the immunoprecipitated kinase activity from TPA- and DMSO-treated HL-60 cells showed a 6-fold increase in TPA-treated cells and a 10-fold increase in DMSO-treated cells after 5 days (Figure 3), a time point at which TPA-treated cultures have stopped dividing and have differentiated into macrophage-like cells (Rovera et al., 1979). By that time most of the DMSO-treated HL-60 cells have differentiated into myelocytes and metamyelocytes (Collins et al., 1978). Morphological changes after the rapid induction of differentiation of
TPA-treated HL-60 cells correlated very well with the time-
dependent increase in kinase activity. Already 48 h after induc-
tion, an elevated kinase activity could be observed which reaches
maximum values at 72 h. The viability of the cells at that time
was 99.9% as measured by trypan blue staining. No signifi-
cant changes were found between days 3 and 5. In comparison
the slower differentiation-inducing effect of DMSO also cor-
responds well with the time-dependent increase in kinase activ-
ity. Using a monoclonal antibody against pp60⁴⁴ (Lipsich et al.,
1983), we could prove that the differential increase in kinase ac-
tivity detected after induction of differentiation of HL-60 cells is
pp60⁴⁴ specific and not due to a crossreaction of the TBR-
sera with other related protein kinases. Quantitation of the auto-
phosphorylation of pp60⁴⁴ from the immune complex kinase
assay shows a 3-fold increase in kinase activity in cells which
have been treated with 10 nM TPA for 3 days and an 8-fold in-
crease in cells which have been treated with 1.25% DMSO for
5 days (Table 1). The change in pp60⁴⁴ kinase activity was also
reflected when pp60⁴⁴ was immune precipitated using the anti-
c-src antibody (Courtneidge, 1985) and enolase was added as
exogenous substrate (Table 1).

To evaluate a regulation of the kinase expression on the tran-
scriptional or post-transcriptional level, we analyzed
poly(A)+ RNA from the untreated cells and those treated with
DMSO for 5 days (Table 1). Quantitation of the increase in
kinase activity in cells which have been treated with 1.25% DMSO
for 5 days shows induction of differentiation and an elevated
pp60⁴⁴ kinase activity similar to the results shown in Figure 1.
This result shows that the observed effect is directly
associated with cellular differentiation and not due to unspecific
interaction of the tumor promoter.

Discussion

The promyelocytic HL-60 cell line has been used extensively as
a model system in studies of tumor cell growth and differentia-
tion processes, since HL-60 cells are bipotential with respect to
myeloid and macrophage differentiation (Collins et al., 1978;
Rovera et al., 1979).

Previous studies have shown that the proto-oncogene c-myc
is amplified in both the original tumor and the cell line as
demonstrated by Southern blot analysis (Dalla-Favera et al.,
1982; Westin et al., 1982). Changes in expression of proto-
oncogenes in HL-60 promyelocytic leukemia cells induced to dif-
ferentiate by TPA or DMSO have been reported for c-myc
(Filmus and Buick, 1985; Grosso and Pitot, 1985; Watanabe
et al., 1984, 1985), c-fos (Mitchell et al., 1985; Müller et al.,
1984, 1985), c-fms (Saribas et al., 1985) and N-ras (Watanabe
et al., 1985; Murry et al., 1983). The results suggest that multiple proto-
oncogenes may be activated during differentiation of HL-60 cells
along both the myeloid and monocytic lineages.

The c-src gene, the cellular homolog of the RSV transforming
gene, is one of the most thoroughly studied proto-oncogenes.
As reported by several groups, the c-src gene product pp60⁴⁴ is
expressed in different cell types. A high expression of c-src
and its gene product is always found in neural tissues (Cotton
and Bragge, 1983; Sorge et al., 1984; Barnekow and Bauer,
1984; Gessler and Barnekow, 1984; Brugge et al., 1985). The
expression of c-src is developmentally regulated (Gessler and
Barnekow, 1984; Scharf and Barnekow, 1984). An increased
expression of the c-src gene product during embryogenesis of
vertebrates coincides with the onset of organogenesis, a finding
which suggests that c-src plays a role in induction or main-
tenance of differentiation processes.
Fig. 2. Two-dimensional thin-layer electrophoresis of 32P-labeled heavy chain of TBR-IgG after precipitation of TPA-treated HL-60 cell extract. P-SER = phosphoserine, P-THR = phosphothreonine, P-TYR = phosphotyrosine. For further details see Materials and methods.

![Figure 2](image)

Fig. 3. Kinetics of pp60\(^{c-src}\) kinase activity in HL-60 cells after induction of differentiation with TPA (upper part, +TPA) and with DMSO (lower part, +DMSO). Experimental details as described in Figure 1.

![Figure 3](image)

**Table I. The kinase activity of pp60\(^{c-src}\) in HL-60 cells (c.p.m.)**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Untreated cells</th>
<th>+10 nM TPA</th>
<th>+1.25% DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enolase</td>
<td>100</td>
<td>350</td>
<td>750</td>
</tr>
<tr>
<td>pp60(^{c-src})</td>
<td>150</td>
<td>450</td>
<td>1250</td>
</tr>
</tbody>
</table>

Standard deviation <5%. The kinase assays and quantitation of the phosphorylation reactions were performed as described in Materials and methods.

Fig. 4. Expression of c-src mRNA after induction of differentiation of HL-60 cells. 4 µg of poly(A)^+ RNA from untreated cells and cells grown in the presence of 10 nM TPA for 3 days or 1.25% v/v DMSO for 5 days, were analyzed by Northern blotting and hybridization to nick-translated v-src fragments. Ribosomal RNA was visualized by ethidium bromide staining.

**Fig. 4**

In the present study we examined the kinetics of pp60\(^{c-src}\) kinase activity during myeloid and macrophage differentiation of HL-60 cells. The results obtained show an activation of tyrosine-specific kinase activity, reactive with pp60\(^{c-src}\) antibodies, during differentiation of the monomyelocytic cells in vitro.

Although the TBR-sera used in this study have previously been shown to react specifically with pp60\(^{c-src}\) (Barnekow and Bauer, 1984; Gessler and Barnekow, 1984), we cannot totally exclude a cross-reaction with other highly related proteins such as the putative c-yes gene product. Since the gene products of the viral counterparts to c-src and c-yes display an 82% sequence homology, it appears likely that the respective cellular gene products will also have structural similarities (Kitamura et al., 1982).

The results from the immune complex kinase assays using the monoclonal antibody directed against pp60\(^{c-src}\) and the antipeptide antibody to pp60\(^{c-src}\) show a good correlation to the data obtained using TBR-sera. A stimulation of the pp60\(^{c-src}\) kinase activity was always observed in differentiation-induced HL-60 cells. Therefore the possibility that it is not pp60\(^{c-src}\) which is being detected seems to be very unlikely.

In previous experiments on the expression of c-src in em-
bryonic chicken tissues we have shown a good correlation between the level of c-src mRNA, the amount of pp60c-src and its kinase activity (Gessler and Barnekow, 1984). In contrast, in this study similar levels of c-src specific mRNA were found in TPA- or DMSO-treated and -untreated HL-60 cells, whereas the pp60c-src kinase activity increased during differentiation processes. These results point to the possibility of a second mechanism to regulate c-src expression. The increase in kinase activity during cellular differentiation may be due either to an increased enzymatic activity of the protein itself, or to an increase in the number of pp60c-src molecules. Regulation of pp60c-src kinase activity by post-translational modifications have been reported recently. Courtenidge (1985) proposed that pp60c-src activity is negatively regulated by C-terminal tyrosine phosphorylation. Bolen et al., (1985) described an association of increased pp60c-src kinase activity in human neuroblastoma cells with tyrosine phosphorylation in the amino-terminal portion of the protein.

Preliminary experiments on c-src protein expression in HL-60 cells before and after induction of differentiation pointed to the possibility that the increase of pp60c-src tyrosine kinase activity may be due to an increase in pp60c-src molecules, which is not paralleled by an increase in the level of c-src mRNA. This specific mechanism of regulation may, however, be restricted to tumor cells or certain differentiation processes.

Recently several groups have shown a TPA-dependent increase in the phosphorylation of pp60c-src and pp60c-src (Tamura et al., 1984; Purchio et al., 1985). TPA, a known activator of protein kinase C, causes phosphorylation of the viral and cellular pp60c-src at serine 12 (Gould et al., 1985). The pp60c-src tyrosyl kinase activity is not affected by this modification (Tamura et al., 1984; Purchio et al., 1985). Our control data with the chick embryo cells are also consistent with results reported by Goldberg et al. (1980) and Pietropaolo et al. (1981). These authors conclude from their experiments that treatment of chick embryo cells with the tumor promoter TPA has little or no effect on the level of protein kinase encoded by the viral nor by the cellular src genes. This largely excludes an interaction of the tumor promoter with the kinase. Therefore the elevated level of pp60c-src kinase activity, detected in HL-60 cells after induction of differentiation, seems to be a differentiation-dependent event. This interpretation is further strengthened by our finding that the increased kinase activity is also detectable in HL-60 cells which were incubated in the presence of 10 nM TPA for about 20 h and subsequently incubated in TPA-free medium for 2 days.

Materials and methods

Reagents

All chemicals used were the purest grade available. Sodium vanadate was purchased from Sigma (Munich, FRG).

Antibodies

Antisera from RSV-tumor-bearing rabbits (TBR-sera) were prepared by simultaneous injection of SR-RSV-D and PR-RSV-C strain into newborn rabbits in a modification (Ziemiecki and Friis, 1980) of the procedure described by Brugge and Erikson (1977). A monoclonal antibody to pp60c-src (Linsch et al., 1983) was obtained from J. Brugge (Stone Brook, USA) and the anti-peptide antibody to pp60c-src (Courtenidge and Smith, 1984) from S. Courtenidge (EMBL, Heidelberg, FRG).

Cells

HL-60 cells were a gift from S. Dube (Yale University, New Haven, CT) and HeLa cells were provided by D. Kübler (German Cancer Center, Heidelberg, FRG). The cell lines were grown in Dulbeccco-Vogt modified Eagle’s minimum essential medium (DMEM) supplemented with 10% fetal calf serum. Chicken embryo cells were prepared from 11-day-old embryos (Lohmann-Tierzucht GmbH, Cuxhaven, FRG) and were maintained in DMEM containing 5% newborn calf serum.

Preparation of cell extracts and immunoprecipitation

Cells were washed, lysed and the extract clarified as described previously (Barnekow and Bauer, 1984). 0.2 mg soluble protein was incubated with 5 µl TBR-serum for at least 60 min at 4°C and precipitated with the protein A-containing bacteria, Staphylococcus aureus, strain Cowan 1. The bacterial bound immunocomplex was washed and the protein kinase assay was carried out by a modification (Barnekow and Bauer, 1984) of the method of Collett and Erikson (1978). Immunocomplex kinase assays using a monoclonal antibody that recognizes mammalian pp60c-src were conducted using cellular lysates adjusted to the same protein concentration (400 µg per reaction). After immunoprecipitation in antibody excess followed by goat anti-mouse IgG (Dianova, Hamburg, FRG), the antigen-antibody complexes were collected by adsorption to protein A-containing bacteria and the kinase assay was carried out as described earlier (Barnekow and Bauer, 1984), except that the phosphorylation reaction was for 10 min at room temperature. Quantitation of the phosphorylated pp60c-src from the immune complex kinase assay was conducted by localization of the pp60c-src by autoradiography, excision of the corresponding gel region, and counting in a liquid scintillation counter.

For the Ap4A experiments, various concentrations of diadenosinetetraphosphate were added to the washed immunoprecipitates 5 min before the kinase reaction was started by addition of γ-32P-ATP.

For quantitative determination of radioactivity incorporated into the heavy chain IgG of the pp60c-src antibody, samples were separated in parallel on a gel and the radioactive IgG heavy chain bands were cut out, solubilized and their radioac-


Activation of pp60+-src kinase during differentiation


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References