

Activation of the pp60^{c-src} kinase during differentiation of monomyelocytic cells *in vitro*

Angelika Barnekow and Manfred Gessler

Institut für Medizinische Virologie der Justus-Liebig-Universität Giessen, Frankfurter Str. 107, D-6300 Giessen, FRG

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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 leukaemia cell line as a model system. Induction of monocytic 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^{c-src} tyrosine kinase, but not with increased *c-src* gene expression. Control experiments exclude an interaction of TPA and DMSO themselves with the pp60^{c-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; Gonda 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; Scharl and Barnekow, 1982; Barnekow and Scharl, 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, pp60^{v-src} (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 (Scharl 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

leukaemia (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^{c-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^{c-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^{c-src} kinase activity from normal cells (Courtneidge, 1985).

HL-60 cells, which in the presence of DMSO differentiate into granulocytes, displayed a differentiation-dependent increase in the expression of pp60^{c-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 pp60^{v-src} kinase activity, but in concentrations up to 100 μ M, 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 μ M (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

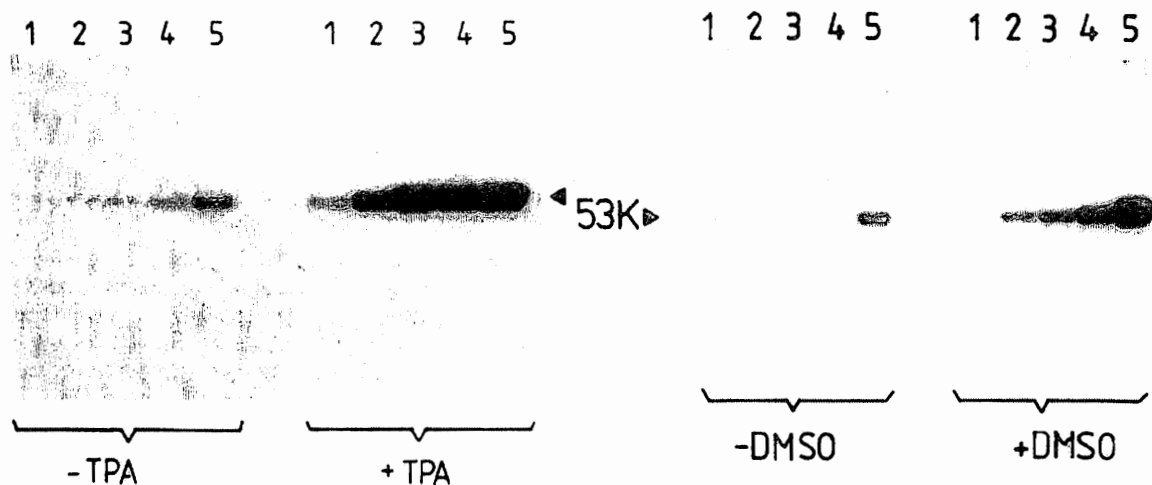


Fig. 1. Demonstration of pp60^{c-src} kinase activity in HL-60 cells after induction of differentiation. 5×10^6 cells were treated with 10 nM TPA or 1.25% DMSO respectively for 1, 2, 3, 4 and 5 days (1-5). In a control experiment untreated sister cultures (-TPA) were tested. Equal amounts of protein were immunoprecipitated with an excess of TBR-serum and subsequently the kinase assay was performed. 53K indicates the heavy chain of the anti-pp60^{c-src} antibody.

TPA-treated HL60 cells correlated very well with the time-dependent increase in kinase activity. Already 48 h after induction, 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 significant changes were found between days 3 and 5. In comparison the slower differentiation-inducing effect of DMSO also corresponds well with the time-dependent increase in kinase activity. Using a monoclonal antibody against pp60^{c-src} (Lipsich *et al.*, 1983), we could prove that the differential increase in kinase activity detected after induction of differentiation of HL-60 cells is pp60^{c-src} specific and not due to a crossreaction of the TBR-sera with other related protein kinases. Quantitation of the autophosphorylation of pp60^{c-src} 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 increase in cells which have been treated with 1.25% DMSO for 5 days (Table I). The change in pp60^{c-src} kinase activity was also reflected when pp60^{c-src} was immune precipitated using the anti-c-src antibody (Courtneidge, 1985) and enolase was added as exogenous substrate (Table I).

To evaluate a regulation of the kinase expression on the transcriptional or post-transcriptional level, we analyzed poly(A)⁺ RNA from the untreated cells and HL-60 cells, which had been treated with TPA for 3 days or with DMSO for 5 days. At these time points the treated cells were shown to express the most elevated amount of kinase activity (Figure 3). Surprisingly, no significant differences could be observed when comparing the amount of c-src specific transcripts in untreated cells and TPA- or DMSO-treated HL-60 cells (Figure 4A and B).

To confirm that the increase in pp60^{c-src} kinase activity is indeed a differentiation-specific phenomenon and not dependent on an interaction of the compounds TPA and DMSO with the kinase itself, we treated the human HeLa cell line and normal chick embryo cells both with TPA and DMSO for the time intervals described in the HL-60 experiments. Neither the HeLa cells (data not shown) nor the chicken cells displayed a time-dependent increase in kinase activity during treatment with TPA and DMSO (Figure 5), which implies that the increased expression of kinase activity in treated HL-60 cells is a differentiation-

dependent event. This statement is supported by the finding that incubation of HL-60 cells in the presence of 10 nM TPA for about 20 h and subsequent incubation of the cultures in TPA-free medium for 2 days, shows induction of differentiation and an elevated pp60^{c-src} 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 differentiation 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 differentiate 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 (Sariban *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 pp60^{c-src} is expressed in different cell types. A high expression of c-src and its gene product is always found in neural tissues (Cotton and Brugge, 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; Schartl 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 maintenance of differentiation processes.

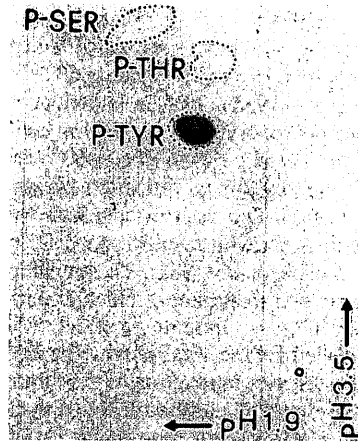


Fig. 2. Two-dimensional thin-layer electrophoresis of ³²P-labeled heavy chain of TBR-IgG after precipitation of TPA-treated HL-60 cell extract. P-SER = phosphoserine, P-THR = phosphothreonine, P-TYR = phosphotyrosine. For further details see Materials and methods.

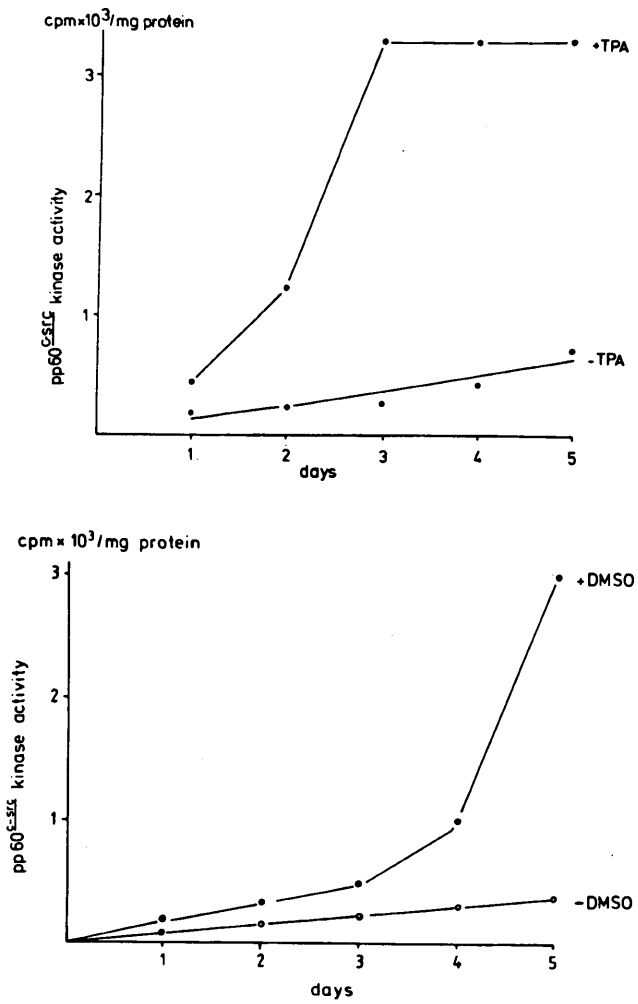


Fig. 3. Kinetics of pp60^{c-src} kinase activity in HL-60 cells after induction of differentiation with TPA (upper part, +TPA) and with DMSO (lower part, +DMSO). In control experiments untreated sister cultures were tested (-TPA, -DMSO). Experimental details as described in Figure 1.

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

Substrate	Untreated cells	+10 nM TPA	+1.25%DMSO
Enolase	100	350	750
pp60 ^{c-src}	150	450	1250

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

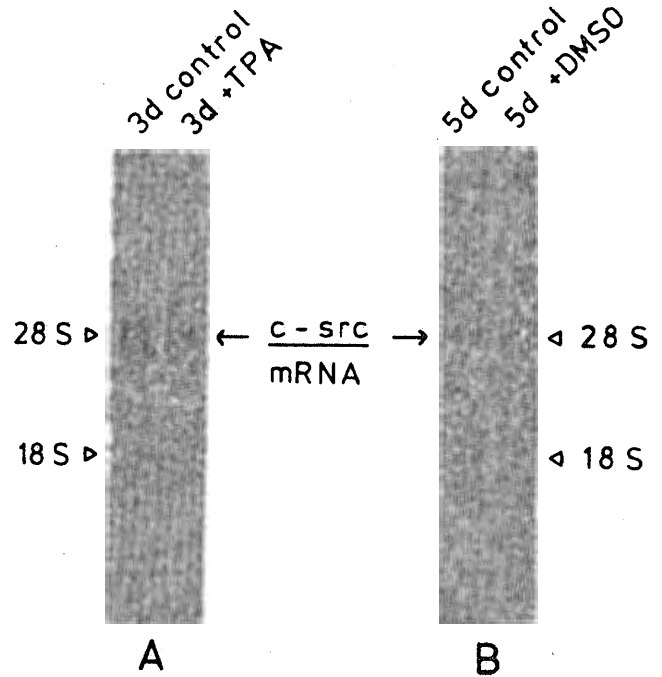


Fig. 4. Expression of *c-src* mRNA after induction of differentiation of HL-60 cells. 4 μg of poly(A)⁺ RNA from untreated cells and cells grown in the presence of 10 nM TPA for 3 days or 1.25% v/v DMSO for 5 days, were analyzed by Northern blotting and hybridization to nick-translated *v-src* fragments.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

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^{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 anti-peptide 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-

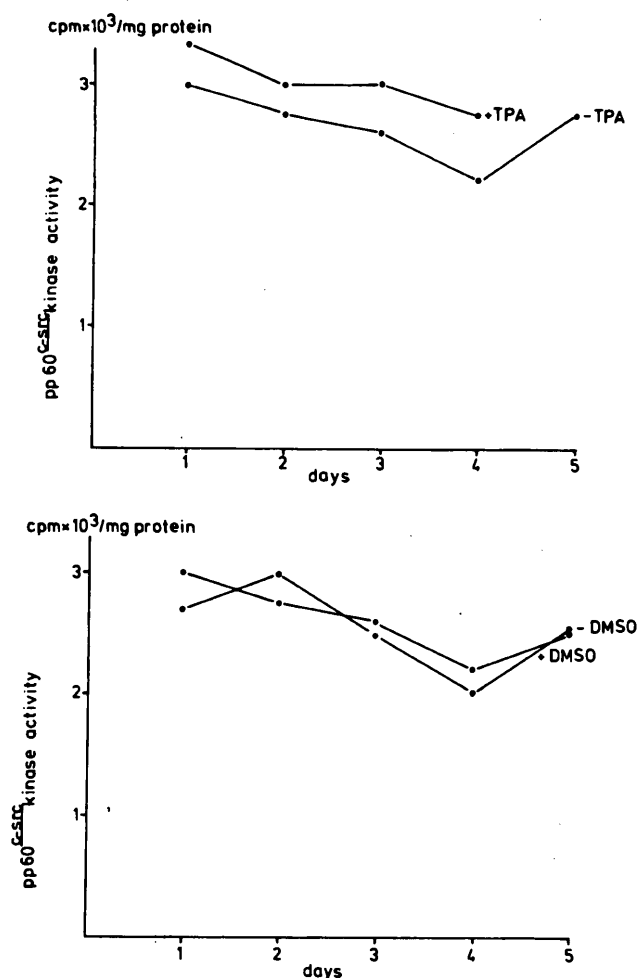


Fig. 5. Kinetics of pp60^{c-src} kinase activity in treated (+TPA, +DMSO) and untreated (-TPA, -DMSO) chick embryo cells. 5×10^6 cells were treated with 10 nM TPA or 1.25% DMSO respectively and processed as described in Figure 1 and Materials and methods.

brionic chicken tissues we have shown a good correlation between the level of *c-src* mRNA, the amount of pp60^{c-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 pp60^{c-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 pp60^{c-src} molecules. Regulation of pp60^{c-src} kinase activity by post-translational modifications have been reported recently. Courtneidge (1985) proposed that pp60^{c-src} activity is negatively regulated by C-terminal tyrosine phosphorylation. Bolen *et al.*, (1985) described an association of increased pp60^{c-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 point to the possibility that the increase of pp60^{c-src} tyrosine kinase activity may be due to an increase in pp60^{c-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 pp60^{v-src} and pp60^{c-src} (Tamura *et al.*, 1984; Purchio *et al.*, 1985). TPA, a known activator of protein kinase C, causes phosphorylation of the viral and cellular pp60^{src} at serine 12 (Gould *et al.*, 1985). The pp60^{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 pp60^{c-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 (Ziemiński and Friis, 1980) of the procedure described by Brugge and Erikson (1977). A monoclonal antibody to pp60^{src} (Lipsich *et al.*, 1983) was obtained from J. Brugge (Stony Brook, USA) and the anti-peptide antibody to pp60^{c-src} (Courtneidge and Smith, 1984) from S. Courtneidge (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 Dulbecco-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 μ l TBR-serum for at least 60 min at 4°C and precipitated with the protein A-containing bacteria, *Staphylococcus aureus*, strain Cowan I. 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 pp60^{c-src} were conducted using cellular lysates adjusted to the same protein concentration (400 μ g 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 pp60^{c-src} from the immune complex kinase assay was conducted by localization of the pp60^{c-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 pp60^{src} antibody, samples were separated in parallel on a gel and the radioactive IgG heavy chain bands were cut out, solubilized and their radioac-

tivity determined by liquid scintillation counting and calculated per mg soluble protein cell extract.

Quantitative kinase assays using enolase as a substrate were performed as described by Courtneidge (1985), after incubation of 0.2 mg cell lysate with 2 μ l of the anti-peptide antibody to pp60^{src}. The region of the Coomassie-blue stained gel slice containing the enolase was excised and counted in a scintillation counter.

Protein determination

Determination of protein concentration in the supernatant of the clarified cell lysates was carried out on trichloroacetic acid-precipitated aliquots according to the method of Lowry *et al.* (1951).

Phosphoamino acid analysis

³²P-labelled IgG was cut out of the gel, eluted from the gel sample and processed for phosphoamino acid analysis as described recently (Barnekow and Bauer, 1984) following the method of Hunter and Sefton (1980).

Isolation and analysis of RNA

Extraction and Northern blot analysis of RNA was performed as described recently (Gessler and Barnekow, 1984). In brief, total RNA was prepared from cultured cells by lysis and repeated precipitation from 7.5 M guanidine hydrochloride, followed by chloroform extraction. After one cycle of oligo-(dT)-cellulose chromatography, 4 μ g of poly(A)⁺RNA were separated on 1.2% formaldehyde/agarose gels and blotted onto Gene Screen membranes. Blots were hybridized with nick-translated PstI fragments, representing the 5' half of SR-RSV-A v-src gene from pSRA-2 (DeLorbe *et al.*, 1980). Hybridization was performed at 43°C in the presence of 50% formamide, followed by washing at 50°C in 1 \times SSC, 0.5% SDS.

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