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The *bcr*-c-*abl* tyrosine kinase activity is extinguished by TPA in K562 leukemia cells

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Tyrosine kinase activity is associated with the transforming potential of several oncogenes. Human chronic myeloid leukemia (CML) cells and cell lines have been shown to contain an active *bcr*-c-*abl* p210 tyrosine kinase as a consequence of the Philadelphia chromosomal translocation. In the present work the activity of the c-*abl* and c-*src* oncogene-encoded tyrosine kinases was investigated during phorbol diester (TPA) induced differentiation of the K562 CML cells. The high tyrosine kinase activity of $p210^{bcr\text{-}c\text{-}abl}$ is strongly reduced during the initial 24 h of TPA treatment. In contrast, the activity of the c-*src* tyrosine kinase is not changed. No change occurs in the expression of the c-*abl*-specific RNAs during this period. Following the reduction of *bcr*-c-*abl* kinase activity, cell proliferation is arrested and megakaryoblastic antigens appear on the cells. Sodium butyrate caused a slight decrease in growth rate and of *bcr*-c-*abl* kinase activity during erythroid differentiation whereas no changes in c-*src* or c-*abl* tyrosine kinase activities were seen in DMSO-treated control cells.

Chronic myeloid leukemia; Oncoprotein; Philadelphia chromosome; Phosphotyrosine; Tyrosine kinase

1. INTRODUCTION

The K562 cells have several erythroid characteristics [1,2], but can be differentiated along either the megakaryoblastic or erythroid lineages [3–5]. Collins and Groudine found that the c-*abl* oncogene sequences in K562 cells are rearranged and amplified, and produce a novel transcript of about 8.5 kb [6–8]. This rearrangement of c-*abl* is typical for CML [8,9], and consists of a replacement of the 5′ c-*abl* oncogene sequences with DNA from the *brc* gene of chromosome 22 due to a translocation of the c-*abl* DNA from chromosome 9 to the Philadelphia chromosome [10,11]. The resulting *bcr*-c-*abl* fusion transcript encodes the $p210^{bcr\text{-}c\text{-}abl}$ protein, which has an enhanced tyrosine kinase activity when compared with the corresponding normal protein $P145^{c\text{-}abl}$ [11,12]. It has been proposed that the fusion protein, which resembles the oncogene-encoded protein of the Abelson murine leukemia virus, is responsible for increased cell proliferation during the chronic phase of CML [13]. However, hematopoietic cells of CML patients undergo nearly normal differentiation until the blast crisis [14]. It is intriguing whether the maturation of CML cells can occur in the presence of the high *bcr*-c-*abl* oncoprotein tyrosine kinase activity. A model to study this question in vitro is provided by the megakaryoblastic and erythroid differentiation of

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Abbreviations: CML, chronic myeloid leukemia; DMSO, dimethyl sulfoxide; TBRS, tumor-bearing rabbit serum; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate

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the K562 leukemia cells induced by TPA and sodium butyrate, respectively.

We have previously shown that the c-*abl* mRNA levels do not change during megakaryoblastic differentiation of K562 cells [15]. Here I report the unexpected finding that the c-*abl* tyrosine kinase activity is strongly down-regulated early during TPA treatment and subsequent differentiation, when there is also an arrest of cell proliferation.

2. MATERIALS AND METHODS

2.1. *Culture and treatment of the cells*

The K562 cells [1] were grown in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum and antibiotics. Differentiation was induced at a cell density of about 3×10^5/ml with TPA (1.6–3 nM, Sigma) dissolved in DMSO or with sodium butyrate (1.2 mM). Control cells were left untreated or treated with DMSO alone.

2.2. *Immunological reagents*

The anti-pEX-2 and anti-pEX-5 rabbit anti-*abl*-protein antibodies [11] were a kind gift from Dr Owen Witte and the Rous sarcoma virus-induced tumor-bearing rabbit serum (TBRS) from Dr Herman Oppermann [16]. Monoclonal anti-*src*-protein antibodies 327 were a kind gift from Dr Joan Brugge [17] and the anti-phosphotyrosine antibodies were from Dr H. Fujio (Osaka University, Japan) [18].

2.3. *Immunoprecipitation and kinase assays*

2×10^7 cells were washed three times with PBS, suspended in 70 μl PBS, and lysed rapidly in cold kinase lysis buffer (1% Triton X-100, 0.05% SDS, 10 mM Na_2HPO_4-NaH_2PO_4, pH 7.0, 150 mM NaCl [9]). Subsequent immunoprecipitation and kinase reactions were performed as described by Konopka and Witte [11,12]. For $pp60^{c\text{-}src}$ kinase assays 20 mM $MgCl_2$ was added to the reaction mixture. [γ-^{32}P]ATP (5–10 μCi/reaction, Amersham RPN 168), together with 0.8 μM unlabeled ATP was added to initiate the kinase reaction which was carried out at 30°C for 10–30 min. Phosphorylated proteins were separated in polyacrylamide gel electrophoresis under reducing conditions. Some autoradiograms were quantitated using densitometric scanning with Ultrascan (LKB, Bromma, Sweden).

3. RESULTS

K562 cells treated with nanomolar concentrations of TPA cease cell division within the first 24 h as shown by cell counting (fig.1A). A decrease in thymidine incorporation is seen within hours after the addition of TPA and by 4 days of treatment is only 7% of control (fig.1B). Flow cytometric analysis of cellular DNA content confirms the virtual absence of cells in the S-phase of cell cycle 4 days after addition of TPA (fig.2). Sodium butyrate-treated cells showed only modestly decreased growth (fig.1). At 2 and 4 days of

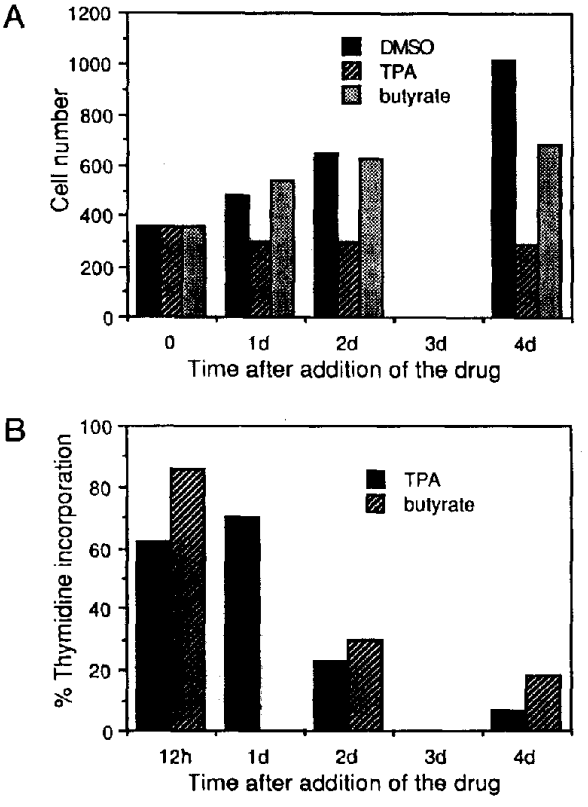

Fig.1. Proliferation (A) and thymidine incorporation (B) of DMSO-, TPA- and butyrate-treated K562 cells. (A) Cell number/μl was counted after trypan blue vital staining. (B) At indicated times a 4 h pulse with 0.4 μCi [^{3}H]thymidine was given and 2×10^5 cells were harvested for measurement of acid-insoluble radioactivity. Thymidine incorporation is expressed as percentage of incorporation compared to cells treated with DMSO for the same time period. Shown are mean values calculated from three separate experiments. Variation between values was less than 10%.

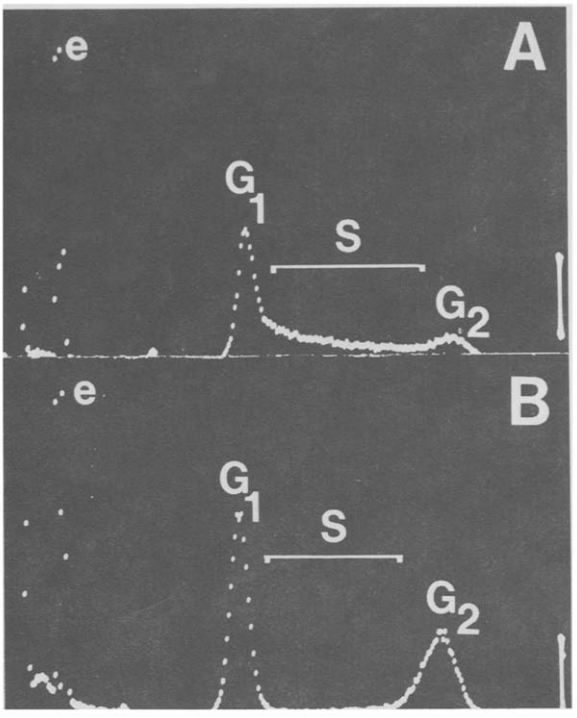

Fig.2. Flow cytometric analysis of the DNA content of DMSO-treated (A) and TPA-treated (B) K562 cells. Cells treated for 4 days with TPA or DMSO were stained with ethidium bromide, and their DNA content was analyzed by FACS IV. Chick erythrocyte nuclease was used as a calibration standard (e). The percentage of cells in the S-phase in (A) is about 56%. In (B), twice the amount of cells shown in (A) were analyzed, to document the virtual absence (less than 20%) of cells in the S-phase.

treatment, respectively, 5 and 10% of nuclease from TPA-induced cells contain more than a diploid amount of DNA suggesting the development of nuclear polyploidy (not shown). As shown by others [3,4] and in our previous work [15] the TPA-induced K562 cells acquired several megakaryoblastic features.

Anti-*abl*-protein immunocomplex kinase reactions from K562 cells treated for 4 days with TPA, sodium butyrate or as a control with DMSO were analysed by polyacrylamide gel electrophoresis. The $p210^{bcr\text{-}c\text{-}abl}$ along with several other polypeptides were phosphorylated in samples from DMSO-treated control cells. In contrast, no phosphoproteins were seen in reaction mixtures from TPA-treated cells. There was only a slight reduction in the kinase activity in samples from sodium butyrate-treated cells in comparison with the control cells. The c-*src* kinase activity recovered from the same samples with TBRS, on the contrary, remains unaltered by either TPA or butyrate treatment (fig.3). A similar result was also obtained using anti-*src* monoclonal antibodies 327 ([17]; not shown).

Anti-phosphotyrosine immunocomplex kinase reactions were analyzed in parallel with the anti-pEX-5 *abl*-protein immunoprecipitates. The results in fig.4 show that antiphosphotyrosine an-

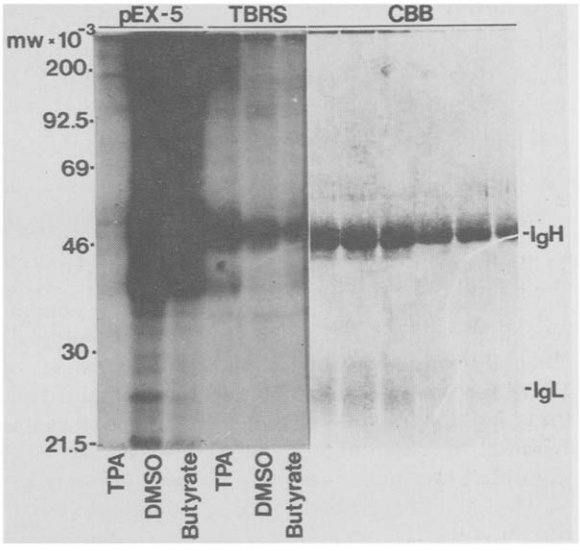

Fig.3. Comparison of the c-*src* and *bcr*-c-*abl* kinase activities in differentiating K562 cells. Immunocomplex kinase assays were performed as described in section 2.3 using anti-pEX-5 antiserum or TBRS respectively, from cells differentiated for 4 days. Samples were separated in 10% polyacrylamide gel and stained with Coomassie brilliant blue (CBB) and subjected to autoradiography. Shown is an 8 h autoradiogram and the stained gel, with molecular mass markers indicated on the left. The positions of the IgG heavy (IgH, 50 kDa) and light (IgL, 25 kDa) chains are indicated on the right. The film was overexposed to document the virtual absence of phosphoproteins in the TPA lane. The major phosphopolypeptides seen in the pEX-5 lanes are better visualized in fig.4. The slight amount of the 210 kDa polypeptide among the TBRS phosphoproteins from TPA-treated cells represents sample leakage from the adjacent pEX-5 lane of butyrate-treated cells.

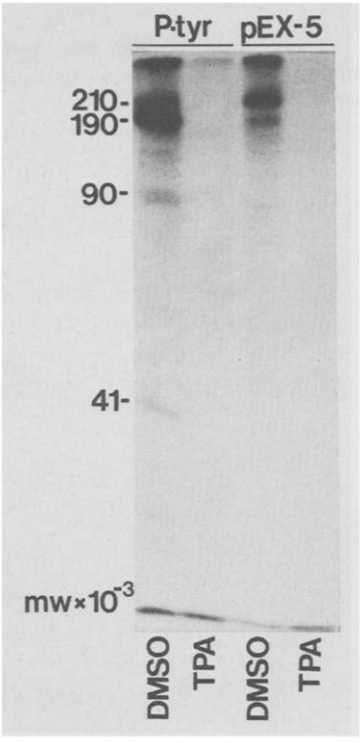

Fig.4. Comparison of kinase activities immunoprecipitated from K562 cells with anti-phosphotyrosine (P-tyr) or with the pEX-5 antibodies. Cells treated with DMSO or TPA for 24 h were immunoprecipitated with the two antisera and analyzed by the kinase assay followed by electrophoresis in a 7.5% gel. In longer exposure lower molecular mass polypeptides were seen in the pEX-5 (DMSO) lane.

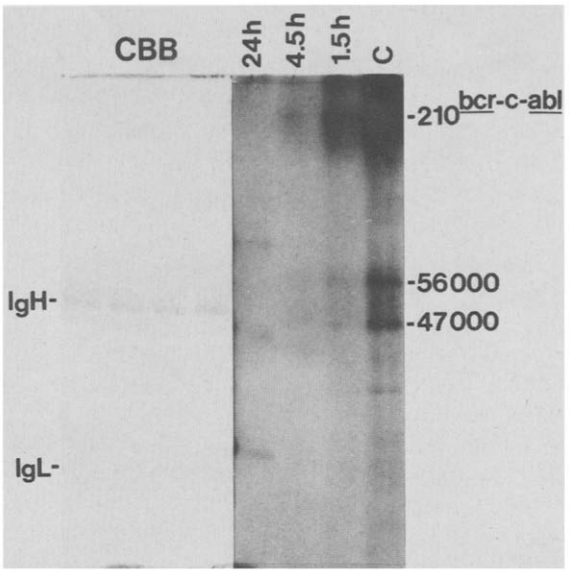

Fig.5. Kinetics of the c-*abl* kinase downregulation during TPA-induced differentiation. Immunocomplex kinase assays were performed using K562 cells treated with TPA for the indicated lengths of time. The proteins were analyzed by electrophoresis and autoradiography as in fig.3. The electrophoretic mobilities of the $p210^{bcr\text{-}c\text{-}abl}$ fusion protein and the 56 and 47 kDa major phosphoproteins are shown. The vague bands seen in the 24 h lane represent leakage of molecular mass markers from the adjacent lane. (C) Control experiment with untreated cells. Other symbols as in fig.3.

tibodies yield a phosphopolypeptide comigrating with the 210 $kDa^{bcr\text{-}c\text{-}abl}$ phosphoprotein. In addition, they yield a polypeptide of 190 kDa (a probable degradation product of p210 [19]) and minor phosphopolypeptides of 90 and 41 kDa apparent molecular masses. This analysis shows that a major tyrosine-phosphorylated protein in untreated K562 cells is the *abl* oncogene-encoded tyrosine kinase, and that the activity of the kinase declines during differentiation as measured by two independent antibodies.

The *bcr*-c-*abl* kinase activity was decreased already 1.5 h after TPA induction and even more at 4.5 h (fig.5). There were practically no proteins phosphorylated in the immunocomplex kinase reaction from cells differentiated for 24 h.

Several other phosphoproteins are seen in the *bcr*-c-*abl* kinase reactions. The most prominent polypeptides, of 56 and 47 kDa are marked in fig.5. The identities of these phosphoproteins are not known. Alkaline treatment of the gels, which has been shown to enrich in phosphotyrosine at the cost of phosphoserine and phosphothreonine [20] resulted in about 50% reduction in the intensity of the radioactive signal from the 56 kDa polypeptide band as well as from the minor 50 kDa polypeptide when measured by densitometric scanning. In contrast, the degree of phosphorylation of the 210, 190 and 47 kDa polypeptides were not altered. Thus, the 56 and 50 kDa phosphoprotein species probably contain a major portion of their phosphate in phosphoserine and phosphothreonine. This was also suggested by their absence from the anti-phosphotyrosine immunoprecipitates.

4. DISCUSSION

Our previous investigations on growth factor and oncogene RNA expression in K562 cells showed that the *bcr*-c-*abl* oncogene RNA persists in K562 cells despite their differentiation and decreased proliferation [15]. Since it was unexpected to find oncogene RNA expression being maintained in differentiating cells, we decided to study the activity of the protein product of the *bcr*-c-*abl* tyrosine kinase. The present results document the virtual absence of tyrosine kinase activity in TPA-treated K562 cells as measured by the immunocomplex kinase assay using two different antisera. We also made a comparison with sodium butyrate-treated K562 cells, which show erythroid differentiation. Only a slight reduction of the kinase activity occurs in these cells.

The immunoprecipitation and immunocomplex kinase experiments with anti-phosphotyrosine antibodies suggest that a major portion of phosphotyrosine in K562 cell polypeptides resides in the *bcr*-c-*abl* kinase. Similar amounts of kinase activity were precipitated with both anti-*abl* and anti-phosphotyrosine antibodies, although the sizes of polypeptides precipitated were somewhat different, probably due to different degrees of proteolytic degradation (see e.g. [19]). The mechanism for the observed difference in the kinase activities of TPA-treated and control cells is unknown. Our immunoprecipitation experiments are too insensitive to detect metabolically labelled *bcr*-c-*abl* protein in K562 cells. Therefore, we are as yet uncertain, whether there is a reduction in the amount of the protein, its kinase activity or in the activities of possible phosphatases in the immunocomplexes. Several other phosphopolypeptides are found in the pEX-5 immunocomplex kinase reactions, when analyzed by polyacrylamide gel electrophoresis. The major phosphopolypeptides of 56 and 50 kDa probably contain most of their radioactive label in phosphoserine and phosphothreonine. These polypeptides may correspond to these reported by Maxwell et al. [21]. Concomitant with the disappearance of the tyrosine kinase-catalyzed phosphorylations, there seems to be a reduction in serine and threonine phosphorylations, possibly by c-*abl*-independent, contaminating kinases. However, the intensity of these polypeptides varied in different experiments and they were not seen in kinase-labeled anti-phosphotyrosine immunoprecipitates.

The c-*src* kinase activity was not detectably affected by TPA treatment. This is particularly interesting, since high levels of the c-*src* kinase and phosphotyrosine are found in platelets [22].

The abnormal *bcr*-c-*abl* tyrosine kinase activity could cause the proliferative advantage of CML cells, such as the cell line K562. It is shown here that during induction of megakaryoblastic differentiation of K562 cells with the protein kinase C activator TPA [23] this tyrosine kinase activity is strongly downregulated and simultaneously the cells stop dividing. Further studies are required to clarify whether and how these events are interrelated.

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