Induction of the 47 kDa platelet substrate of protein kinase C during differentiation of HL-60 cells

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Immunoblot analysis showed that the 47 kDa platelet substrate of protein kinase C (P47) was expressed at low levels in undifferentiated HL-60 leukaemia cells. Treatment of these cells with dimethyl sulphoxide, $1\alpha,25$ -dihydroxycholecalciferol or retinoic acid caused progressive increases in P47 content. Retinoic acid ($1\ \mu\text{M}$) elicited the largest response, a 4-fold increase in P47 protein after 7 days that was accompanied by an increase in translatable P47 mRNA. The induction of P47 by retinoic acid preceded cessation of cell proliferation and development of the capacity to reduce Nitro Blue Tetrazolium, indicating that its expression is an early event in the myeloid differentiation of HL-60 cells.

INTRODUCTION

Agonists that stimulate the hydrolysis of phosphoinositides by phospholipase C generate two second messengers, i.e. diacylglycerol, which activates protein kinase C, and IP_3 , which mobilizes Ca^{2+} ions from the endoplasmic reticulum [1,2]. In the platelet, activation of phospholipase C by thrombin or collagen leads to the phosphorylation of a 47 kDa protein (P47) by protein kinase C [3–8]. Although phosphorylation of this protein is usually associated with platelet degranulation [4], the two phenomena can be dissociated [9]. It has been variously suggested that P47 is lipocortin [10] or the soluble platelet IP_3 5-phosphatase [11]. In the former case, phosphorylation of P47 might enhance the agonist-induced release of arachidonic acid by decreasing its anti-phospholipase A_2 activity [10]. However, if P47 is an IP_3 5-phosphatase that is activated by protein kinase C [11], it might attenuate Ca^{2+} mobilization by hydrolysing IP_3 .

A polyclonal antibody to human platelet P47 has been used to demonstrate the presence of this protein in some permanent human cell lines (M. L. McCaw, J. A. Lynham, C. S. Sartori, M. M. L. Davidson, M. Culty & R. J. Haslam, unpublished work). A low concentration of P47 was found in HL-60 cells, a promyelocytic leukaemia cell line established by Collins *et al.* [12]. These cells differentiate towards neutrophilic granulocytes on prolonged exposure to DMSO or retinoic acid [13,14]. Active phorbol esters such as TPA, on the other hand, induce differentiation into macrophage-like cells [15], and the vitamin D_3 metabolite, $1,25(\text{OH})_2\text{D}_3$, elicits a mixed myelomonocytic phenotype [16]. Maturation of HL-60 cells requires differentiation of pathways of signal transduction, as well as of structural components of the cell. Thus induction of a myeloid phenotype leads to expression of receptors for chemotactic peptides, such as fMLP [17], and an enhanced protein kinase C activity [18]. Addition of fMLP to the differentiated cells

activates phospholipase C and so stimulates protein kinase C, the formation of IP_3 and the mobilization of Ca^{2+} ions, events that probably mediate functional responses, such as superoxide formation and lysosomal enzyme secretion [19–21]. In addition, neutrophilic differentiation of HL-60 cells enhances the activation of phospholipase A_2 and synthesis of platelet-activating factor caused by fMLP or ionophore A23187 [22]. Because studies on platelets have suggested that P47 may play a role in these mechanisms of signal transduction, we have studied the expression of this protein during differentiation of HL-60 cells.

EXPERIMENTAL

Materials

^{125}I was obtained from Chedoke–McMaster Hospitals (Hamilton, Ont., Canada) and ^{35}S methionine ($1400\ \text{Ci/mmol}$) from Amersham (Oakville, Ont., Canada). Nitrocellulose sheets ($0.45\ \mu\text{m}$) were supplied by Bio-Rad Laboratories (Mississauga, Ont., Canada). Retinoic acid (all-*trans*- β), TPA, NBT, chicken egg-white lysozyme and Protein A were obtained from Sigma (St. Louis, MO, U.S.A.), and $1,25(\text{OH})_2\text{D}_3$ was generously given by Dr. J. D. Harvey of Hoffmann–La Roche, Etobicoke, Ont., Canada.

Cell culture

HL-60 cells from the American Type Culture Collection were grown as described elsewhere [23]. Experiments were initiated with cells at a density of $2 \times 10^5/\text{ml}$, duplicate cultures being used for each experimental point. Retinoic acid, TPA and $1,25(\text{OH})_2\text{D}_3$ were added as stock solutions in ethanol so that the final ethanol concentration was no greater than 0.1% (v/v), which had no effect on cell growth or differentiation. DMSO was added directly to cultures at 1.3% (v/v). Cells were counted in a Coulter Counter. As an index of cell proliferation, the cell number in cultures exposed to

Abbreviations: P47, 47 kDa platelet substrate of protein kinase C; IP_3 , inositol 1,4,5-trisphosphate; DMSO, dimethyl sulphoxide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; $1,25(\text{OH})_2\text{D}_3$, $1\alpha,25$ -dihydroxycholecalciferol; fMLP, formyl-methionyl-leucyl-phenylalanine; NBT, Nitro Blue Tetrazolium.

inducers was expressed as a percentage of that in untreated control cultures incubated for 7 days. Neutrophilic differentiation was assessed by TPA-induced superoxide formation, as indicated by the percentage of cells showing NBT reduction [24]. Secreted lysozyme (activity expressed as μg of commercial enzyme) was measured as an index of monocytic differentiation [25].

Measurement of P47 by immunoblotting

HL-60 cells were washed twice in ice-cold phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 , pH 7.4) and samples were precipitated with 10% (w/v) trichloroacetic acid. Protein was determined [26], and 80 μg portions were subjected to SDS/polyacrylamide-gel electrophoresis, with 13% acrylamide in the separating gel [6]. P47 standards (10–100 ng) were electrophoresed in parallel. Non-phosphorylated human platelet P47 was purified by modification of the method described in [6] and used to generate a rabbit anti-P47 serum (M. L. McCaw, J. A. Lynham, C. S. Sartori, M. M. L. Davidson, M. Culty & R. J. Haslam, unpublished work). Electrophoretic transfer of protein to nitrocellulose membranes was carried out as described by Erickson *et al.* [27]. Non-specific binding sites were blocked by shaking with phosphate-buffered saline (154 mM-NaCl/2.0 mM- NaH_2PO_4 , adjusted to pH 7.2 with NaOH) containing 5% (w/v) non-fat milk powder [28] and 0.3% (w/v) Tween 20. The membranes were then incubated with a 1:100 dilution of rabbit anti-P47 serum in the same solution. Protein A iodinated with ^{125}I [29] was used to detect bound antibody, located by autoradiography, cut out and counted for ^{125}I in a γ -radiation counter. The background on each immunoblot was determined by counting radioactivity in two areas that showed no specific immunoreactivity. The P47 content of each sample was estimated from the ^{125}I

associated with the P47 standards, which gave a linear response on each blot. P47 induction (fold increase) was determined relative to protein samples from undifferentiated cells electrophoresed on the same gel.

Translation of HL-60 RNA *in vitro* and immunoprecipitation of ^{35}S -labelled P47

Total RNA was isolated from HL-60 cells, translated in a rabbit reticulocyte lysate system containing [^{35}S]methionine and immunoprecipitated, as described elsewhere [30]. Either 25 μg of total RNA or 0.5 μg of polyadenylated RNA [31], quantified by ethidium bromide fluorescence at pH 8.1 [32], was translated. Samples of translation product containing equal amounts of trichloroacetic acid-precipitable ^{35}S were immunoprecipitated with either 20 μg of the IgG fraction of anti-P47 serum or 10 μl of immune or pre-immune serum. Competition experiments were carried out identically, except that 2 μg of purified platelet P47 was added before immunoprecipitation. Precipitated protein was reduced and alkylated before electrophoresis on an SDS/polyacrylamide gradient gel containing 7–15% acrylamide [30]. ^{35}S -labelled proteins were detected by fluorography [30]. P47 had a slightly lower apparent molecular mass in this system than observed previously [5,6].

RESULTS AND DISCUSSION

Differentiation of HL-60 cells induced by retinoic acid, DMSO or 1,25(OH) $_2$ D $_3$, all of which greatly increased the proportion of cells capable of reducing NBT, was associated with a markedly enhanced expression of P47 (Fig. 1). Cells containing large amounts of P47 also exhibited traces of a related 42 kDa immunoreactive protein that is observed in platelets and co-purifies with P47. Prolonged exposure of the autoradiographs revealed the presence of 10–20 kDa immunoreactive polypeptides

Fig. 1. Expression of P47 in HL-60 cells induced to differentiate by various agents

Cells were subcultured without additions and with 1 μM -retinoic acid, 1.3% DMSO, 100 nM-1,25(OH) $_2$ D $_3$ and 10 nM-TPA, in each case for the period indicated. Cellular protein (80 μg) from each subculture was electrophoresed, blotted on to nitrocellulose and incubated with anti-P47 serum, followed by ^{125}I -labelled Protein A, as described in the Experimental section. P47 standards (10, 30 and 100 ng) were processed identically. An autoradiograph of a representative immunoblot is shown. Values for HL-60 P47 were calculated from the ^{125}I present. Corresponding values for differentiation markers and cell proliferation (means of duplicate determinations) are shown below each lane of the autoradiograph.

in cells induced with retinoic acid; these may be proteolytic fragments of P47. None of the above species were detected when pre-immune serum was used. In contrast with $1,25(\text{OH})_2\text{D}_3$, which induces a mixed myelomonocytic phenotype [16], TPA elicited a monocytic differentiation that was not consistently accompanied by increases in P47 detectable after 4 days (Fig. 1). However, TPA did induce the appearance of small amounts of 10–20 kDa polypeptide, suggesting that it may stimulate both the synthesis and the degradation of P47 (results not shown). Untreated cells grown for 7 days without subculture contained amounts of P47 similar to those in untreated exponential-phase cells, despite density arrest of cell proliferation (Fig. 1). Thus increased expression of P47 in HL-60 cells may be related to the induction of specific differentiated characteristics.

The dose-response relationship and kinetics of induction of P47 were studied by using retinoic acid (Fig. 2). When HL-60 cells were exposed to the compound for 7 days, maximal induction was observed at $1\text{ }\mu\text{M}$, which caused a 4.0 ± 0.5 -fold increase in P47 (mean $\pm$ S.E.M., 3 expts.). Under these conditions, P47 accounted for about 0.07% of total cellular protein. Up to 2-fold increases in P47 were detected with concentrations of retinoic acid that had minimal effects on cell proliferation or differentiation. The time course of P47 expression in response to $1\text{ }\mu\text{M}$ -retinoic acid indicated that induction of P47 was an early event in the differentiation of HL-60 cells (Fig. 2b). P47 began to increase by 1 day and reached

3.1 ± 0.1 -fold its initial amount (mean $\pm$ S.E.M., 3 expts.) after 3 days, well before cell division ceased and the cells gained the ability to reduce NBT. Thus P47 reached a high concentration before the cells were irreversibly committed to differentiation [24]. Expression of P47 reached a peak between 5 and 7 days, and then declined as cell proliferation ceased and cell viability decreased (Fig. 2b). A similar early induction of P47 was observed with DMSO and $1,25(\text{OH})_2\text{D}_3$ (results not shown).

To study P47 mRNA during retinoic acid-induced differentiation, total RNA was isolated after various periods and translated *in vitro*. The translation products were immunoprecipitated with anti-P47 antibody and the precipitate was analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 3a, lanes b–g). The results show that P47 mRNA accounted for a progressively increasing proportion of the total translatable mRNA in differentiating HL-60 cells. An increase in the relative amount of P47 mRNA was even observed between 7 and 9 days, which contrasts with the decrease in P47 protein (Figs. 3a and 2b). However, the former result could have been due to loss of other mRNA species. The initial accumulation of P47 mRNA may have involved increased transcription or the stabilization of mRNA transcribed to low extents both before and after induction of differentiation. Both modes of control have been described for other genes expressed during HL-60 differentiation [33,34].

Several translated proteins in addition to P47 were

Fig. 2. Dose-response relationship and time course of P47 induction by retinoic acid

(a) Cells were subcultured for 7 days with the indicated concentrations of retinoic acid. P47 in the cells was assayed in duplicate immunoblots and expressed in multiples of that found in untreated cells subcultured for 7 days. NBT reduction and cell proliferation are also means of duplicate determinations. To indicate cell proliferation, the numbers of cells in subcultures containing retinoic acid were expressed as percentages of the numbers of cells in subcultures without the compound. (b) Cells were subcultured with $1\text{ }\mu\text{M}$ -retinoic acid for the indicated periods. P47 is expressed in multiples of that found in cells harvested at 0 days (mean values $\pm$ S.E.M. from three determinations). NBT reduction and cell proliferation are means of duplicate determinations. As an index of cell proliferation, the numbers of cells present at various times after subculture with retinoic acid were expressed as percentages of the numbers of cells present after subculture without retinoic acid for 7 days. ■, P47; ●, NBT reduction; ▲, cell proliferation.

Fig. 3. Time course of P47 mRNA induction in HL-60 cells induced to differentiate by retinoic acid

(a) Fluorograph of an SDS/polyacrylamide gel showing the ^{35}S -labelled proteins precipitated by immune IgG from translation *in vitro* of total cellular RNA (the cells used were subcultured at the same time as those in Fig. 2b): a, protein precipitated from a reticulocyte lysate control containing no exogenous RNA; b–g, immunoprecipitated protein corresponding to RNA from cells subcultured with $1\ \mu\text{M}$ -retinoic acid for 0, 1, 3, 5, 7 and 9 days respectively. (b) Fluorograph of a gel containing protein from control immunoprecipitations: a and b, translation products of total RNA (from cells exposed to retinoic acid for 1 day) precipitated with pre-immune serum and immune serum respectively; c and d, translation products of polyadenylated RNA (from cells exposed to retinoic acid for 5 days) precipitated with immune IgG in the presence and absence of $2\ \mu\text{g}$ of platelet P47 respectively; e, as d, with $1\ \mu\text{g}$ of P47 added just before electrophoresis. The molecular masses of standard proteins [30] electrophoresed in parallel are shown on the left of each fluorograph, and the position of platelet P47 is shown on the right.

immunoprecipitated by P47 antibody. These proteins were not derived from the reticulocyte lysate (Fig. 2a, lane a) and were precipitated by pre-immune serum (Fig. 3b, lane a). It is not clear why these proteins do not appear on immunoblots with either immune or pre-immune serum. The specificity of the anti-P47 serum was demonstrated by the absence of translated P47 from precipitations with pre-immune serum (Fig. 3b, lane a) and the competitive effect of added platelet P47 (Fig. 3b, lane c). HL-60-cell P47 that had been translated *in vitro* and purified platelet P47 co-migrated, indicating that P47 was not subject to post-translational modification detectable by gel electrophoresis.

The early induction of P47 during the differentiation of HL-60 cells is consistent with a role for this protein either in commitment of the cells to exit from the cell cycle or in the maturation of a pathway of signal transduction utilized by the differentiated cell. Further progress in understanding the function of this protein in HL-60 cells requires resolution of the conflicting views advanced as to its identity [10,11]. If P47 is lipocortin [10], its enhanced expression, together with that of protein kinase C [18], could play a role in the regulation of phospholipase A_2 , which is more readily activated in differentiated HL-60 cells [22]. However, since our anti-P47 antibody does not bind to recombinant human lipocortin I, and P47 migrates more slowly than lipocortin on SDS/polyacrylamide gels (M. L. McCaw, J. A. Lynham, C. S. Sartori, M. M. L. Davidson, M. Culty & R. J. Haslam, unpublished work), it is unlikely that P47 and lipocortin are related. If, on the other hand, P47 is an IP_3 5-phosphatase [11], possible functions of the protein in differentiating HL-60 cells

include attenuation of the Ca^{2+} signals involved in cell proliferation [2] and/or differentiated functions, such as secretion [19,20].

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