Antisense oligodeoxynucleotide inhibition of δ protein kinase C expression accelerates induced differentiation of murine erythroleukaemia cells

Anna PESSINO, Mario PASSALACQUA, Bianca SPARATORE,* Mauro PATRONE, Edon MELLONI and Sandro PONTREMOLI

Institute of Biological Chemistry, University of Genova, Viale Benedetto XV no. 1, 16132 Genova, Italy

The potential regulatory role of δ protein kinase C (δ PKC) in murine erythroleukaemia cell differentiation was studied by using antisense oligodeoxynucleotides targeting the translation initiation region of mouse δ PKC mRNA. Cell treatment with antisense oligonucleotides, at a concentration of 20 μ M, followed by hexamethylenebisacetamide induction, produced a specific 2-fold increase in the differentiation rate of both slowly and rapidly differentiating murine erythroleukaemia cell clones. Cell permeabilization by a cationic lipid resulted in a decrease of one order of magnitude in the amounts of antisense oligonucleotides necessary to elicit the maximal response, and accelerated the

kinetics of the stimulatory effect. These changes in murine erythroleukaemia cell differentiation rates, observed in both cell clones, were associated with 60 % and 50 % decreases, respectively, in δ PKC immunoreactive protein in slowly and rapidly differentiating cells. The present results indicate strongly that basal levels of δ PKC in murine erythroleukaemia cells are essential in regulating the initial differentiation rate of these cells in response to chemical induction, and provide further evidence that this PKC isoform plays a fundamental role in maintaining the undifferentiated phenotype of murine erythroleukaemia cells.

INTRODUCTION

Murine erythroleukaemia (MEL) cells are a well studied model system of cellular differentiation [1]. These cells undergo a multistep process leading to their commitment to terminal erythroid differentiation when exposed to a variety of chemical agents, among which hexamethylenebisacetamide (HMBA) is one of the most potent [2]. A series of metabolic modifications occur during the latent period preceding irreversible commitment to differentiation. Among the most prominent changes are modifications of protein kinase C (PKC) activity, which undergoes an early and progressive decline after the addition of the chemical inducer [3]. We have recently demonstrated that MEL cells express five different PKC isoforms: α , δ , ϵ , ζ and θ PKC [4]. These isoenzymes display different sensitivities to HMBA-induced down-regulation, δ PKC being one of the more rapidly and extensively degraded isoenzymes. We have also observed that the overall down-regulation is more rapid in an MEL cell clone named C44, selected on the basis of a mild vincristin resistance, displaying a faster differentiation rate and a higher sensitivity to HMBA compared with parental MEL cell line DS19 and with another clone derived from it, named N23, showing differentiation characteristics very similar to DS19 cells [5].

The study of mRNA concentrations of the various PKC isoenzymes in different MEL cell clones, characterized by distinct differentiation rates in response to HMBA, has led us to the observation that rapidly differentiating C44 cells display significantly lower levels of the Ca^{2+} -independent isoenzymes (δ , ϵ , ζ and θ), whereas they express higher concentrations of the only Ca^{2+} -dependent isoform, α PKC [4]. We have focused our interest on δ PKC isoenzyme, which is the PKC isoform most represented in MEL cells. We have observed that the difference in δ PKC mRNA expression levels between N23 and C44 cells is paralleled by a similar difference in δ PKC protein intracellular concen-

trations. Moreover, incorporation of partially purified δ PKC into permeabilized MEL cells causes a delayed onset of HMBA-induced differentiation [6]. On the basis of these findings, as well as of the above-mentioned observation that δ PKC down-regulation in response to HMBA is prominent relative to the other PKC isoenzymes, we have reasoned that basal δ PKC levels may be important in modulating the initial differentiation rate of MEL cells after HMBA exposure.

In the present study we have used antisense oligonucleotides specific for δ PKC mRNA to gain further insight into the involvement of this isoenzyme in the early events after exposure of MEL cells to HMBA that lead to cell commitment to terminal differentiation. For this purpose, we have synthesized a 16-mer oligonucleotide designed to hybridize to the region of the AUG initiation codon of δ PKC mRNA, because this site has been shown to be the optimal target in many studies. Here we describe results that demonstrate that the decrease of δ PKC intracellular concentration, induced by specific antisense oligonucleotide treatment before incubation with HMBA, causes an acceleration of differentiation of both N23 and C44 MEL cell clones.

MATERIALS AND METHODS

Cell culture

MEL cell clones N23 and C44 were obtained and cultured as previously described [7]. Cell differentiation was induced by addition of 5 mM HMBA to the culture medium at a cell density of 10^5 per ml. At the indicated times, the proportion of differentiated cells was assayed by the benzidine reaction, as described in [8]. Briefly, aliquots of cells from suspension culture were centrifuged and resuspended in 20 μ l PBS, pH 7.5. Benzidine staining was performed by adding to cell suspensions equal volumes of 0.2 % benzidine in 0.5 M acetic acid containing 1 % (v/v) hydrogen peroxide. After 1 min incubation, cells were

Abbreviations used: ASN δ , oligonucleotide, antisense oligonucleotide specific for δ protein kinase C; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate; HMBA, hexamethylenebisacetamide; MEL, murine erythroleukaemia; PKC, protein kinase C; RT-PCR, reverse transcriptase–polymerase chain reaction.

* To whom correspondence should be addressed.

deposited onto glass slides and scored for the presence of haemoglobin (blue coloration) by examination with an inverted microscope.

Oligonucleotide treatment of cells

Phosphodiester oligodeoxynucleotides were purchased from TIB Mol. Biol. (Genova, Italy). Antisense oligodeoxynucleotide (5'-AGGGTGCCATGATGGA) was complementary to the translation initiation region (nucleotides -6 to 10) of mRNA specific for mouse δ PKC [9]. Sense (5'-TCGATCATGGCACCCT) and missense (5'-CGAGTAGTTAGAGCGG) oligodeoxynucleotides were used as controls. N23 and C44 cells were washed three times with α -MEM containing 10% (w/v) fetal calf serum previously heated at 65 °C for 30 min to remove most of the DNase activity, and seeded in the same medium in 96-well plates (2×10^4 cells in 200 μ l). Oligodeoxynucleotides were solubilized in culture medium, sterilized by filtration through 0.2 μ m cellulose acetate filters, and added to cell cultures at the indicated concentrations. Incubations were performed for the indicated times. When the incubation was longer than 24 h, culture medium was replaced daily with fresh medium containing all the additions. At the indicated times, cell density was determined and 10^4 cells were induced with 5 mM HMBA in 100 μ l of culture medium. The remaining cells were collected for quantification of δ PKC protein and mRNA. In experiments in which the oligodeoxynucleotides were added in combination with the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N*,*N*-trimethylammonium methylsulphate (DOTAP; Boehringer Mannheim), N23 and C44 cells were seeded in 24-well plates (10^6 cells in 1 ml). Each indicated oligodeoxynucleotide amount was preincubated with 10 μ g DOTAP for 15 min at room temperature in 20 mM Hepes, pH 7.4, and then the mixture was added to individual wells. Twenty-four hours later, cell concentration was determined and aliquots of cells were induced to differentiate.

Immunoblot analysis of δ PKC isoenzymes in MEL cells

At the end of incubation with oligodeoxynucleotides, aliquots of N23 and C44 cells were washed three times with PBS, and equal amounts of cells were resuspended in 60 mM Tris/HCl, pH 6.8, containing 10% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol and 2% (w/v) SDS (Laemmli sample buffer) and heated for 5 min at 95 °C. Samples were then subjected to SDS/PAGE (8% gel), followed by transfer onto pure nitrocellulose membranes (Hybond-C, Amersham). δ PKC isotype was identified by using a specific anti-peptide antibody at 1:5000 dilution (R&D, Berkeley, CA, U.S.A.). Nitrocellulose filters were subsequently probed with an actin-specific polyclonal antibody at 1:200 dilution (Sigma), and actin immunoreactive signal was used as an internal control for normalization of δ PKC amounts. Both immunoreactive proteins were detected by using 125 I-labelled Protein A (Amersham). Radioactive bands were revealed by autoradiography and corresponding areas of the filters were excised and quantified in a gamma counter. Non-specific radioactivity was evaluated by counting non-significant areas of the filter and subtracting them from each determination.

Immune complex δ PKC assay

N23 and C44 cells (2×10^7 cells) were incubated with 1 μ M of either antisense or missense oligonucleotides in the presence of DOTAP, as described previously. After 24 h, cells were washed twice with PBS and lysed in 2 ml of ice-cold lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% (v/v) NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing 2 mM PMSF and 10 μ g/ml

leupeptin. After incubation on ice for 15 min, cells were disrupted by repeated aspiration through a 21-gauge needle and the lysates were centrifuged at 20000 *g* for 15 min. Anti-peptide polyclonal antibodies (2 μ g of immunoglobulins) specific for either δ PKC or α PKC (Santa Cruz Laboratories) were added separately to 1 ml of supernatant and incubated for 2 h at 4 °C on a rotary wheel. Protein A-agarose (50 μ l) was added to all samples and incubation was continued for 1 h. Immunoprecipitates were washed three times with lysis buffer, and twice with 20 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, and immediately used for kinase assays performed *in vitro* according to a modification of the method described in [9]. The assays of δ PKC activity were performed in 0.2 ml of 20 mM Tris/HCl, pH 7.5, containing 100 mM NaCl, 5 mM MgCl₂, 1 μ g phosphatidylserine, 0.4 μ g dioleoin, 10 μ M ATP, 2 μ Ci [γ - 32 P]ATP and 10 μ g histone H1. The activity of α PKC was measured under the same experimental conditions, except that 0.5 mM CaCl₂ was added to the reaction mixture and 10 μ g histone H1 was used as substrate. All kinase reactions were incubated for 10 min at 30 °C, stopped by addition of Laemmli sample buffer and subsequently loaded onto an SDS/12.5% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Blue, dried and exposed to an autoradiographic film for 24 h. The relative intensity of the radioactive bands was evaluated by densitometric scanning.

Chromatographic purification of δ PKC and assay of kinase activity in the presence of oligonucleotides

δ PKC was purified by sequential DEAE-cellulose and hydroxyapatite chromatography, as previously described [10]. Aliquots (50 μ l) of the eluted fractions were resolved by SDS/PAGE (8% gel), followed by immunoblotting for δ PKC isotype detection, as described above. The assay of δ PKC activity was performed with the fractions containing δ PKC immunoreactive protein, by using myelin basic protein oligopeptide 4-14 as a substrate, as previously described [11], in the presence or absence of the indicated amounts of antisense or missense oligodeoxynucleotides.

Quantification of δ PKC mRNA intracellular concentrations

Total RNA was extracted from 10^4 cells from both MEL cell clones by RNeasy total RNA kit (Qiagen). Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to measure the amounts of δ PKC isoenzyme transcript in both cell clones, co-amplified with a β -actin internal control, as previously described [6]. Briefly, reverse transcription of approximately 1 μ g of total RNA from N23 and C44 cells, respectively, was performed with both δ PKC and β -actin antisense oligonucleotides, the sequences of which are specified in [6], as primers (10 pmol each) in a final volume of 20 μ l. Second-strand cDNA synthesis and further amplification were conducted by adding the corresponding [32 P]-labelled sense primers (10 pmol each, 0.5×10^5 c.p.m. per pmol) and 2.5 units Taq DNA polymerase (100 μ l final volume). The PCR was performed for 20 cycles under conditions described previously [10]. The expected amplified fragments were 247 bp (δ PKC) and 324 bp (β -actin) long. To minimize tube-to-tube variation, each experiment was performed in triplicate. Portions (10 μ l) of each reaction were subjected to electrophoresis on a 10% polyacrylamide/7 M urea gel, gel lanes were subsequently sliced and radioactivity was measured in a scintillation counter. Preliminary experiments were conducted to establish conditions that would permit quantification of the starting material. All the determinations were performed within the exponential part of the amplification reaction (at the 20th cycle), and the amplification efficiencies of each set of reactions were found to be comparable.

RESULTS

Effect of δ PKC antisense oligonucleotide treatment on HMBA-induced differentiation of MEL cells

MEL cell clones C44 and N23 were treated with antisense oligonucleotides specific for the translation initiation region of δ PKC mRNA (ASN δ oligonucleotides) at concentrations of up to 30 μ M. Equal concentrations of sense or missense oligonucleotides were added to individual sets of control cells. These amounts of oligonucleotide did not affect cell growth, although higher concentrations (50 μ M) caused a slight non-specific inhibition of cell growth. After 4 days of incubation with oligonucleotides, aliquots of cells were induced to differentiate by addition of HMBA, and the differentiated phenotype was assayed as specified in Materials and Methods. Slowly differentiating

Figure 1 Effect of increasing concentration of antisense oligonucleotide specific for δ PKC on MEL cell differentiation

N23 (a) and C44 cells (b) were incubated with the indicated concentrations of antisense (●), sense (▲) and missense oligonucleotides (◆) for 4 days, as described in Materials and Methods. Every 24 h, medium was replaced with fresh medium containing the appropriate concentration of each oligonucleotide. At the end of the incubation, aliquots of cells were collected and induced to differentiate by the addition of 5 mM HMBA. The percentage of differentiated cells was measured 16 h (C44 clone) and 72 h (N23 clone) after induction. Results are expressed as means $\pm$ S.D. ($n = 4$).

Table 1 Effect of antisense oligonucleotides specific for δ PKC on differentiation in MEL cell clones

MEL cell clones N23 and C44 were treated with either antisense oligonucleotides specific for δ PKC (ASN δ) or missense oligonucleotides (MISS δ) at a final concentration of 20 μ M, and were induced to differentiate by the addition of HMBA, as specified in the Materials and methods section. At the indicated times, cell differentiation was measured as the proportion of benzidine-reactive cells, as described in the Materials and methods section.

Clone N23			Clone C44		
Benzidine-reactive cells (%)			Benzidine-reactive cells (%)		
Time (h)	ASN δ	MISS δ	Time (h)	ASN δ	MISS δ
72	10.2	5.2	24	7.2	3.5
96	44	25	36	61	45
120	88	82	48	95	97
144	99	98			

N23 cells treated with ASN δ oligonucleotide displayed a concentration-dependent increase in the differentiation rate over that of control cells. The maximal response to ASN δ oligonucleotide, at each concentration tested, was observed 72 h after chemical induction, when only approx. 5 % of control cells were differentiated (Figure 1a). The greatest effect of ASN δ oligonucleotide (approx. 2-fold increase) was obtained at a concentration of 20 μ M (IC_{50} 6–8 μ M), whereas sense and missense oligonucleotides did not significantly affect N23 cell differentiation in comparison with untreated cells. Similarly, rapidly differentiating C44 cells showed an acceleration of HMBA-induced differentiation on ASN δ oligonucleotide treatment which was maximal at early times (16 h) after induction (Figure 1b), and the greatest effect was obtained with 20 μ M antisense oligonucleotide (IC_{50} 4–6 μ M). The magnitude of the maximal response was essentially the same as that achieved in N23 cells (2.2-fold increase). Sense and missense oligonucleotides had no effect on C44 cell differentiation kinetics relative to untreated cells. The effect of antisense oligonucleotide treatment on both N23 and C44 cell clones persisted as differentiation proceeded, becoming less appreciable when the differentiation approached 100 % (Table 1).

In time-course experiments, maximal acceleration of differentiation of N23 cells was obtained by treatment with 20 μ M ASN δ oligonucleotide for 3 days (Figure 2a). No further increase was observed with longer incubation times. Surprisingly, the increase in differentiation rate stimulated by ASN δ in C44 cells was already remarkable after 1 day of oligonucleotide treatment and was maximal after 2 days of incubation with ASN δ (Figure 2b).

Effect of cell permeabilization with DOTAP on ASN δ oligonucleotide-mediated acceleration of MEL cell differentiation

The use of cationic lipids has been reported to increase the potency of antisense oligonucleotides in selectively inhibiting the expression of specific genes [12,13]. Most of the effect of these charged lipids relates to their ability to bind oligonucleotides and enhance cellular uptake by fusing with the cell membrane [14].

Figure 2 Time course of the effect of antisense oligonucleotide specific for δ PKC on MEL cell differentiation

N23 (a) and C44 cells (b) were incubated with antisense (●), sense (▲) and missense (◆) oligonucleotides, at a concentration of 20 μ M, for the indicated times, as described in Materials and methods. At the end of the incubation, aliquots of cells were collected and induced to differentiate by adding 5 mM HMBA. The percentage of differentiated cells was measured 16 h (C44 clone) and 72 h (N23 clone) after induction. Results are expressed as means $\pm$ S.D. ($n = 4$).

Figure 3 Effect of the combination of antisense oligonucleotide specific for δ PKC and DOTAP on MEL cell differentiation

N23 (a) and C44 cells (b) were incubated with the indicated concentrations of antisense (●) and missense (○) oligonucleotides in the presence of DOTAP (10 μ g) for 24 h, as described in Materials and methods. At the end of the incubation, medium was replaced with fresh medium containing 5 mM HMBA. The percentage of differentiated cells was measured 16 h (C44 clone) and 72 h (N23 clone) after induction. Results are expressed as means $\pm$ S.D. ($n = 3$).

We sought to use the cationic lipid DOTAP in combination with ASN δ oligonucleotides to investigate whether the magnitude and/or the kinetics of ASN δ oligonucleotide effect on MEL cell differentiation might be affected. Preliminary experiments demonstrated that DOTAP concentrations of up to 10 μ g/ml did not affect cell viability and differentiation rate (not shown).

Figure 3 illustrates the results of a 24 h exposure of N23 and C44 cells to a combination of various concentrations of ASN δ oligonucleotide and DOTAP at 10 μ g/ml, followed by HMBA induction. Under these experimental conditions, both MEL cell clones were responsive to ASN δ oligonucleotide concentrations more than one order of magnitude lower than those necessary to elicit an effect in the absence of the cationic lipid, probably as a result of an increased uptake of oligonucleotides by cells. C44 cells showed the maximal effect when exposed to 0.5 μ M ASN δ oligonucleotide (IC_{50} 0.2 μ M), whereas twice the amount of oligonucleotide was necessary to obtain the maximal activity in N23 cells (IC_{50} 0.5 μ M). The maximal accelerations of differentiation in response to antisense oligonucleotide treatment in the presence of DOTAP were 2.5- and 2.7-fold in N23 and C44 cells respectively, only slightly greater than the maximal effects achieved with ASN δ at 20 μ M in the absence of DOTAP. However, it is noteworthy that, in the presence of the cationic lipid, N23 cells became maximally responsive to ASN δ oligonucleotide after only 24 h instead of 3 days of pre-incubation.

Mechanism of action of ASN δ oligonucleotide

N23 and C44 cells were analysed for their δ PKC content after exposure to various concentrations of ASN δ oligonucleotide in the presence or absence of DOTAP. The same oligonucleotide-treated cells from which aliquots were removed to be induced to differentiate were subjected to immunoblotting with polyclonal antibodies specific for δ PKC. The same immunoblot was subsequently probed with an anti-actin antibody and actin immunoreactive signal was used as a standard to normalize the δ PKC signal. In both cell lines, the increases in differentiation rates

Figure 4 Specific inhibition of δ PKC protein expression in MEL cell clones by antisense oligonucleotide

N23 (a) and C44 (b) cells were incubated in the absence or presence of antisense or missense oligonucleotides (20 μ M) for 2 days (C44 cells) or 3 days (N23 cells). Aliquots of cells were collected and subjected to SDS/PAGE (8% gel), followed by immunoblotting with antipeptide antibodies specific for δ PKC, and, subsequently, with polyclonal antibodies against actin. Immunoreactive bands were revealed with 125 I-labelled Protein A. Specific radioactivity associated with actin (open bars) and δ PKC (hatched bars) immunoreactive bands was measured in a gamma counter. In the insets, autoradiographs of representative immunoblot assays performed with N23 (a, inset) and C44 cells (b, inset) are shown. C, control untreated cells; M, missense oligonucleotide-treated cells; A, antisense oligonucleotide-treated cells.

promoted by ASN δ oligonucleotide treatment were paralleled by a specific inhibition of δ PKC expression. In the absence of DOTAP, incubation of cells with ASN δ at the maximal concentration of 20 μ M resulted in a decrease of δ PKC immunoreactive protein to about 40–45% of the control value in N23 cells (Figure 4a) and to 50% of the control value in C44 cells (Figure 4b). Simultaneous incubation of cells with DOTAP and 1 μ M ASN δ obtained essentially the same effects (not shown). Occasionally, the δ PKC immunoreactive band appeared as a doublet, with apparent molecular masses of 74 and 76 kDa, as reported in several other studies [15,16]. When this occurred, the total levels of both δ PKC subspecies were markedly reduced after ASN δ oligonucleotide treatment.

To verify that the decrease in δ PKC protein mediated by ASN δ oligonucleotide treatment was associated with a corresponding decrease in the kinase activity, δ PKC was specifically immunoprecipitated from either N23 or C44 cell clones exposed to 1 μ M ASN δ oligonucleotide in the presence of DOTAP, and kinase assays were performed on the immunoprecipitates, with histone H1 as a substrate. As shown in Figure 5, antisense treatment of either cell clone resulted in a remarkable decrease in histone H1 phosphorylation, in comparison with missense-treated control cells. The decrease in phosphate incorporation into histone H1 was approx. 55–65% in both antisense-treated cell clones, in two separate experiments. In contrast, the activity of the other major PKC isoenzyme expressed in MEL cells, δ PKC, was unaffected by ASN δ oligonucleotide treatment, as measured in similar immune complex kinase assays (see Materials and Methods; results not shown).

In addition, because a recent study showed that oligonucleotides are potent inhibitors of β 1PKC *in vitro* [17], we thought it of interest to verify whether changes in δ PKC activity caused by ASN δ oligonucleotide might be responsible for part of

Figure 5 Immune complex assay of PKC activity in antisense-treated MEL cell clones

N23 (a) and C44 cells (b) were incubated with 1 μ M antisense or missense oligonucleotides in the presence of DOTAP for 24 h. At the end of the incubation, cells were lysed, and the lysates were subjected to immunoprecipitation with antipeptide antibodies specific for δ PKC, as specified in Materials and Methods. Kinase reactions were performed on the immunoprecipitated pellets, as described in Materials and Methods, with histone H1 as a substrate. The reaction mixtures were electrophoresed on an SDS/12.5% polyacrylamide gel, which was subsequently exposed to an autoradiographic film. The arrow indicates phosphorylated histone H1.

Figure 6 Levels of δ PKC mRNA in MEL cell clones after treatment with δ PKC antisense oligonucleotide

Total RNA was extracted from N23 (a) and C44 cells (b) (10^5 cells) previously exposed to 20 μ M antisense or missense oligonucleotides, for 2 days (C44 cells) or 3 days (N23 cells). RNA was subjected to RT-PCR by using primers specific for δ PKC and β -actin, as described in Materials and Methods. The 32 P-labelled amplification products corresponding to δ PKC (247 bp) and β -actin (324 bp) gene transcripts were electrophoretically resolved on a 7 M urea/10% polyacrylamide gel, which was then subjected to autoradiography. The radioactive bands were excised from the gel and radioactivity was measured in a scintillation counter.

the specific effect of this oligomer on the differentiation kinetics of MEL cell clones. Partially purified δ PKC was incubated with ASN δ or the corresponding missense oligonucleotide at concentrations of up to 50 μ M and kinase activity was assayed by measuring 32 P incorporation into myelin basic protein oligopeptide 4–14 in the presence of cardiolipin. None of the oligonucleotide concentrations were effective in producing any modification of δ PKC catalytic activity relative to control, ruling out the potential involvement of aptameric inhibition of δ PKC in ASN oligonucleotide mechanism of action (not shown).

To gain information about the molecular mechanism responsible for inhibition of δ PKC expression, we studied the effect of ASN δ oligonucleotide on δ PKC mRNA concentrations in N23 and C44 cells. δ PKC mRNA levels were measured by co-amplification of δ PKC gene product with an internal control transcript, β -actin, by means of a quantitative RT-PCR assay. Because ASN δ oligonucleotide did not change δ PKC mRNA levels, with or without DOTAP (Figure 6), the inhibitory effects on protein expression are probably at the level of translational initiation.

DISCUSSION

Oligonucleotides complementary to specific mRNA regions have been used to inhibit the synthesis of a number of cellular proteins

in vivo [14,18,19]. The mechanisms by which these antisense oligonucleotides produce their specific effect may depend on the structure of target mRNA, the specific site of the mRNA to which they hybridize, the cell type, the chemical characteristics of the oligonucleotide itself, and presumably other still unidentified factors. The proposed mechanisms of action include stimulation of mRNA degradation by RNase H, inhibition of new protein synthesis by translational arrest, prevention of mRNA maturation and transport out of the nucleus and inhibition of gene transcription by formation of a triple helix with DNA [14].

In this study we have used antisense techniques to examine the role of δ PKC in HMBA-induced MEL cell differentiation. This PKC isoform is the major subspecies expressed in MEL cells [4]. Moreover, it is a good candidate for a regulatory role on the onset of MEL cell differentiation, because we observed that partially purified δ PKC caused a decrease in differentiation rates when introduced into lysolecithin-permeabilized MEL cells [6]. Here we demonstrate in a direct and specific way that δ PKC basal concentration in MEL cells plays a fundamental role in the early events of HMBA-induced differentiation.

Exposure of N23 and C44 cells to ASN δ oligonucleotide was followed by a 60% and 50% decrease in δ PKC expression, respectively. The effect of antisense oligonucleotides was specific in that no change in protein levels was observed when a missense version of the same oligonucleotide or the corresponding sense oligonucleotide was used in the incubation media. In antisense-treated cells, the reduction of PKC protein levels was associated with a similar decrease in the kinase activity, as measured in immune-complex kinase assays *in vitro*. These specific decreases in δ PKC expression and activity caused an acceleration of HMBA-induced differentiation that was very similar (approx. 2–2.5-fold) in the two cell clones, even though their differentiation kinetics are significantly different. Nevertheless, differences exist between the responses of the two cell clones to ASN δ oligonucleotides. In the absence of the cationic lipid DOTAP, ASN δ oligonucleotide-treated C44 cells exhibited the maximal effect on differentiation after 48 h treatment, in comparison with the 72 h necessary to obtain the optimal stimulation in the N23 clone. In the presence of DOTAP, both clones were responsive to ASN δ oligonucleotide after 24 h treatment. Nevertheless, 0.5 μ M antisense oligonucleotide was sufficient to produce the greatest effect in C44 cells, whereas double that amount of ASN δ oligonucleotide was maximally effective in the N23 clone. The reason for these discrepancies is unclear, but there are several possible explanations. N23 cells express 2–3-fold higher levels of δ PKC than C44 cells. Thus inhibition of δ PKC expression may require higher ASN δ oligonucleotide concentrations and longer incubation times. Another tentative explanation is a potentially lower membrane permeability to oligonucleotides of N23 cells, which may be partly overcome by the use of cationic lipids.

Recent evidence has been provided for a role of oligonucleotides as aptamers, i.e. nucleic acid molecules that bind to certain proteins in a partly or totally sequence-independent way [17,20,21]. Bock et al. [20] have demonstrated that a panel of oligomers containing a repetitive GGTT consensus sequence could bind purified thrombin and inhibit its catalytic activity at nanomolar concentrations. Another recent study [17] showed that oligonucleotides (both phosphodiester and phosphorothioate oligodeoxynucleotides) inhibit purified β 1PKC, independently of their sequence. Here we have shown that oligonucleotides have no effect on δ PKC activity in assay *in vitro*; thus aptameric inhibition of this serine–threonine kinase is not involved in mediating the effect of ASN δ oligonucleotide.

Other studies have reported that antisense oligonucleotide treatment of intact cells resulted in a decrease in target mRNA

concentration [14]. Here we have shown that the decrease in protein expression measured in ASN δ oligonucleotide-treated cells was not accompanied by any change in δ PKC mRNA levels. This is in agreement with previous observations by Chiang et al. [12], who reported that an antisense oligonucleotide targeting the translation initiation site of ICAM-1 is highly effective in inhibiting protein synthesis without affecting mRNA levels. A possible explanation for this effect is that oligonucleotide-mediated inhibition of protein synthesis occurs, in this case, via translational arrest of the corresponding mRNA.

Preliminary experiments have been performed by treating N23 and C44 cells with antisense oligonucleotides complementary to the translation initiation regions of the other PKC isoforms displaying lower expression levels in C44 cells (ϵ , θ and ζ PKC) [4]. So far, only ζ PKC antisense oligonucleotide produced a specific decline in ζ PKC protein (approx. 30% of the control value) in both cell clones; however, this decrease was not associated with any alteration of HMBA-induced differentiation, suggesting that this isoenzyme is not prominently involved in the regulation of this process. We were unable to obtain significant inhibition of the expression of ϵ and θ PKC by treating MEL cells with antisense oligonucleotides complementary to the AUG translation initiation sites of the corresponding mRNAs. Despite the fact that this mRNA region is considered to be highly susceptible to antisense inhibition, previous studies have demonstrated that, in particular cases, other sites on the mRNA (e.g. the 3' untranslated region) can be better targets for antisense oligonucleotide action [12]. Work is in progress to test whether antisense oligonucleotides designed to hybridize to other sites of ϵ and θ PKC mRNAs are better inhibitors of the expression of these isoenzymes.

The identification of the molecular mechanism by which δ PKC regulates MEL cell differentiation awaits further investigation. Increasing evidence has been provided by several studies supporting a specific role of δ PKC in cell cycle control. Overexpression of this PKC isoenzyme in CHO fibroblasts (unlike that of other PKC subspecies, such as α , β 2 and ζ PKC) specifically resulted in the arrest of cytokinesis after cell exposure to phorbol esters, indicating δ PKC as a candidate for one of the serine-threonine kinases regulating the cell cycle [22]. A number of studies reported that either alteration of the phosphorylation state or changes in intracellular levels of δ PKC affect opposite but correlated processes such as cell transformation and differentiation. δ PKC was found tyrosine phosphorylated in murine keratinocytes that expressed oncogenic *v-rasHa*, and produced benign tumours *in vivo* [23]. Also, in 32D haematopoietic cells and NIH-3T3 fibroblasts, both overexpressing δ PKC, this serine-threonine kinase was found tyrosine phosphorylated in response to phorbol ester treatment [24]. Tyrosine phosphorylation of purified δ PKC was achieved *in vitro* by src tyrosine kinase [24,25]. This covalent modification resulted in an increase of δ PKC activity [24,25], although some experimental evidence seemed to argue otherwise [23]. Increased levels of δ PKC, as well as of α PKC, were found associated alternatively with transformed cells or with cells with restored differentiation capacity [26,27]. These apparent discrepancies in the effects of elevated levels of PKC isoenzymes in different cell lines may indicate that these PKC isotypes serve cell-type-specific functions, which may vary according to the available cell machinery.

In conclusion, our results point to δ PKC as a critical determinant of MEL cell differentiation. In particular, it seems that sustained δ PKC levels and/or activity are essential in maintaining

the undifferentiated phenotype of MEL cells. In previous studies we have shown that extensive down-regulation of this enzyme is already noticeable 4 h after HMBA treatment of N23 cells, a time largely preceding irreversible commitment to terminal differentiation of these cells. Moreover, the present study demonstrates that decreased expression of δ PKC by specific antisense oligonucleotide treatment causes an acceleration of HMBA-induced differentiation of both N23 and C44 cell clones. Taken together, these findings strengthen the hypothesis that a decrease in δ PKC levels and/or activity is an essential prerequisite for overcoming the molecular block to differentiation in MEL cells.

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