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Gsα AVAILABILITY TO CHOLERA TOXIN-CATALYSED ADP-RIBOSYLATION IS DECREASED IN MEMBRANES OF RETINOIC ACID-TREATED LEUKEMIC CELL LINES HL-60 AND THP-1

A POSTTRANSLATIONAL EFFECT

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Abstract—Retinoic acid (RA) induces HL-60 and THP-1 leukemic cell lines to differentiate into granulocyte-like and monocyte-like cells. Limited data are available concerning the effects of RA on components of the cyclic AMP pathway in human myeloid leukemic cells. We showed previously a decrease in adenylate cyclase activity in the presence of histamine, prostaglandin E1 and forskolin in RA-treated HL-60 cells as compared to untreated cells. We examined the elements of the signal transduction pathway utilized by RA in the human myeloid cell line HL-60 and the human monocytic cell line THP-1. We therefore studied the effect of RA on the activity of the stimulatory G-protein (Gs). We demonstrate that addition of RA to two human myeloid leukemia cell lines, HL-60 and THP-1, does not induce a reduction of the 2 subunit of Gs (Gsα) RNA or Gsα protein in the plasma membrane but leads to a rapid decrease in the cholera toxin (CTX)-catalysed ADP-ribosylation of Gsα. In addition, this effect seems to be specific to RA, since there was no modification in Gsα ADP-ribosylation in the membranes of cells treated with dimethyl sulfoxide (DMSO), another inducer of differentiation in HL-60 cells.

The human myeloid leukemia cell line HL-60 differentiates to morphologically and functionally mature granulocytes in the presence of all-*trans*-retinoic acid (RA¶), the active metabolite of vitamin A [1]. Recently, it has been shown that this agent induces the *in vivo* differentiation of leukemic blasts from patients with acute promyelocytic leukemia, leading to complete remissions [2, 3].

The molecular mechanism by which RA induces differentiation still remains unknown. In some cell types, its pharmacological action has been linked to its binding to a specific cytoplasmic protein, CRABP, which would presumably act as a transporter to the nucleus [4]. However all assays failed to detect a CRABP in leukemic cells [5]. Recently, several nuclear proteins with binding specificity for RA have been characterized but their exact role in the differentiation process has not yet been established [6, 7].

We have shown previously that the response of adenylate cyclase to known effectors such as forskolin, histamine and prostaglandin E1 was diminished significantly after RA-induced differentiation in intact HL-60 and THP-1 cells [8]. We, therefore, analysed in the present report the effect of RA on the availability for CTX-catalysed ADP-ribosylation of Gs, which activates the catalytic subunit of adenylate cyclase [9–11]. G-proteins are heterotrimeric membrane proteins composed of α, β and γ subunits. Gsα couples stimulatory hormone receptors to adenylate cyclase and is a substrate for CTX-catalysed ADP-ribosylation in the presence of NAD^+ [12]. We have demonstrated here that addition of RA to two human myeloid leukemia cell lines, HL-60 and THP-1, does not induce a reduction of Gsα RNA or protein in the plasma membrane but leads to a rapid decrease in the CTX-catalysed ADP-ribosylation of Gsα. Finally, this event seems to be specific to RA since DMSO, which induced HL-60 differentiation, did not modify ADP ribosylation of Gsα.

MATERIALS AND METHODS

Chemicals. RA was provided by Dr Bollag (Hoffman-La Roche, Basel, Switzerland). RMPI was from Gibco (Uxbridge, U.K.). Fetal bovine serum was from Boerhinger Mannheim (F.R.G.). CTX and IAP were from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

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¶ Abbreviations: RA, all *trans*-retinoic acid; DMSO, dimethyl sulphoxide; G-protein, GTP-binding protein; GS, the G-protein which stimulates adenylate cyclase (Gs); Gsα, the α subunit of Gs; C, control; CTX, cholera toxin; IAP, pertussis toxin; CRABP, cytosolic retinoic acid binding protein; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GADPH, glyceraldehyde-3-phosphate deshydrogenase.

Radioactive NAD (30 Ci/mmol) was from Du Pont-New England Nuclear (Boston, MA, U.S.A.), ^{125}I protein A (30 Ci/mg) and [α^{32}P]dCTP (3000 Ci/mmol) were from Amersham International, U.K.

Antiserum was provided by Dr G. Milligan (University of Glasgow, U.K.) All other reagents were of the highest grade commercially available.

Cell lines. HL-60 [13] and THP-1 [14] cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 I.U./mL penicillin and 100 μg/mL streptomycin at a concentration of 2×10^5 cells/mL in a 5% CO_2 humidified atmosphere at 37°. Cell viability was determined by Trypan blue exclusion and found to be always above 90%.

Differentiation of both cell lines was induced by treatment with RA for 5 days. RA was dissolved in absolute ethanol at an initial concentration of 10^{-2} M, aliquoted and stored at −80°. Dilutions in culture medium were prepared immediately before each experiment. A final concentration of 10^{-6} M and 10^{-5} M RA was used to differentiate HL-60 and THP-1 cells, respectively. The extent of differentiation was assessed by morphology, non-specific esterase staining and NBT reduction testing. Under these conditions, RA induced the differentiation of HL-60 and THP-1 cells to granulocytic-like and monocytic-like cells, respectively.

Isolation of RNA and RNA blotting. Total RNA was isolated from control and RA-treated cells by guanidine isothiocyanate solubilization and centrifugation over a CsCl cushion [15].

For Northern analysis, 10 μg of total RNA were resolved on an 0.8% agarose gel and then transferred to a Nylon/Hybond C filter. Filters were prehybridized at 42° in prehybridation solution (50% deionized formamide, 5 × SSC [1 × = 150 mM NaCl, 15 mM sodium citrate, 5 × Denhardt's solution (5 × Ficoll, BSA and polyvinylpyrrolidone, each at 1 mg/mL) 0.1% SDS and 250 mg/mL boiled salmon sperm DNA] overnight. Hybridization was performed in prehybridization solution containing 10% dextran sulfate and the ^{32}P-labelled probe (Gsα) [16] at 42° for 12 hr. Following hybridization, filters were washed at 20° in 2 × SSC and 0.1% SDS, four times for 5 min, and at 50° in 0.2 × SSC and 0.1% SDS, two times for 15 min, before exposure to X-ray films. The cDNA insert fragment for Gsα (and GAPDH) was labelled to high specific activity (10^6 cpm/mL) using a random primer labelling kit (Amersham Multiprime Kit). Human 28s and 18s tRNAs were used as markers.

Membrane preparation and NAD labelling. Control and RA-treated cells were cultured for 5 days (unless otherwise specified) and then washed in phosphate buffer. The cell membranes were prepared as described previously [17]. Membranes were suspended in 1 mL potassium phosphate buffer pH 7.3, disrupted with a polytron homogenizer (Kinematica, Luzerne, Switzerland), using three bursts of 15 sec each at 4°, and centrifuged at 600 *g* for 10 min at 4°. Plasma membrane preparations were obtained from low-speed supernatant centrifugation at 20,000 *g* (15 min, 4°). The resulting pellets were resuspended in 100 μL of 50 mM potassium phosphate buffer containing 20 mM thymidine, 5 mM ADP ribose, 20 mM arginine, 1 mM ATP, pH 7.3. Membrane extracts (100 μL) in this buffer were incubated with 100 μM GTP and 10 μM [α^{32}P]NAD in the presence of CTX (25 μg/mL) for 30 min at 30°. CTX was preactivated by incubation with 20 mM dithiothreitol for 10 min at 30° immediately before use. The reaction was stopped by the addition of 1 mL of ice-cold potassium phosphate buffer and centrifugation at 20,000 *g* for 15 min at 4°. The pellets were solubilized by addition of 75 μL Lubrol 12A9 to a final concentration of 0.7% in 2.5 mM Hepes, 0.25 mM $MgCl_2$, 0.125 mM EDTA, pH 8, as described previously [17].

SDS–PAGE. Lubrol extracts of cell membrane proteins (50 μg proteins) were prepared for SDS gel electrophoresis by adding SDS and 2β mercaptoethanol to a final concentration of 1% (w/v) and 5% (v/v), respectively. SDS gel electrophoresis was performed according to Laemmli [18] using 10% polyacrylamide gels. The gels were stained by Coomassie blue, dried and exposed to X-ray film (Kodak X Omat) for 12 hr at −80°. Quantification of the ^{32}P-radiolabelled bands was performed by laser densitometry (2202 Ultroscan, LKB).

Immunoblotting with Gsα antiserum. Electrophoresed samples (50 μg membrane proteins) were transferred to nitrocellular filter and blotted with antibody (1/300 dilution) against Gsα. The Gsα antiserum (CSI) was obtained from rabbits after repeated immunization with a conjugate Keyhole Limpets Hemocyanin and a synthetic peptide corresponding to the RMHLRQYELL C terminal sequence of Gsα (this antibody is characterized elsewhere) [19, 20].

Blots were washed with buffer containing 5% (wt/vol.) fat-free dried milk (Regilait, France), 0.2% Nonidet P40, 0.025% sodium azide, 50 mM Tris–HCl (pH 8), 2 mM $CaCl_2$ and 80 mM NaCl, incubated for 1 hr with Gsα antisera in the same buffer and then washed for 20 min three times. Antibody binding was detected by incubation of the blots with ^{125}I protein A (Amersham, 100,000 cpm/mL) in the same buffer. The blots were then washed and dried before autoradiography [21].

RESULTS

To ascertain whether a change in the amount of Gsα might be responsible for the decreased enzyme activity and cAMP accumulation in differentiated cells, expression of Gsα mRNA transcripts, expression of Gsα polypeptide and CTX-catalysed ADP-ribosylation of HL-60 and THP-1 cells, before and after RA-induced differentiation, were examined.

We examined the steady state mRNA level of GSα in control and RA-differentiated HL-60 and THP-1 cells. In these experiments the integrity of the mRNA was confirmed by formamide/agarose gel electrophoreses prior to measurement. A small amount of a 2.0 kb Gsα transcript was detected by RNA blot analysis of HL-60 and THP-1 cells. Figure 1 shows that RNA levels were not modified in differentiated HL-60 and THP-1 cells as compared to the levels detected in untreated cells. GAPDH is

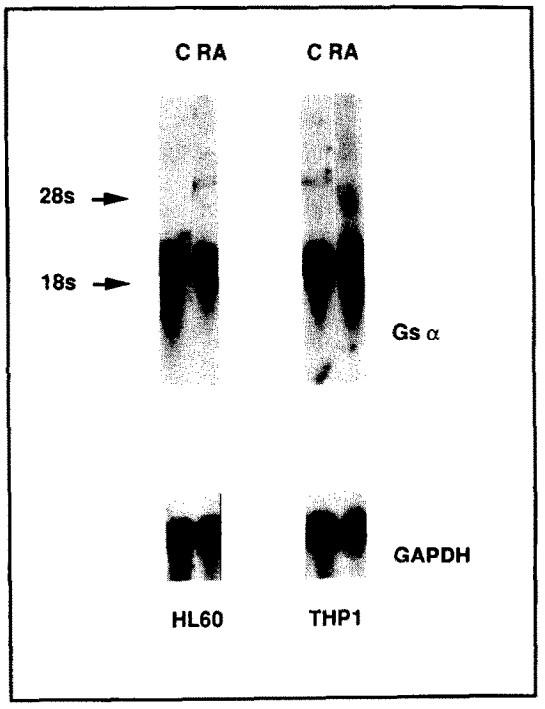

Fig. 1. Expression of Gsα transcripts in HL-60 and THP-1 cells. HL-60 (left) and THP-1 (right) cells were treated for 5 days with 10^{-6} M and 10^{-5} M RA, respectively. The cells were then lysed, RNA was purified and total RNA (10 μg per lane) was analysed by Northern Blot as described in Materials and Methods. The cDNA probes were for Gsα [1] and GAPDH [2]. GADPH was used as a marker for RNA level.

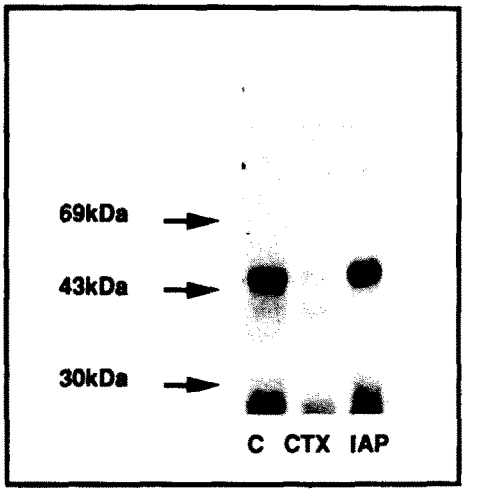

Fig. 2. CTX-dependent ADP-ribosylation of membrane components of HL-60 cells. Membranes from untreated HL-60 cells (left) and HL-60 cells pretreated with 10^{-8} M CTX (middle) or IAP (right) for 24 hr were ADP-ribosylated in the presence of [α^{32}P]NAD and 25 μg/mL activated CTX as described under Materials and Methods. The proteins were separated by SDS–12% PAGE, dried and autoradiographed overnight at −80°. The apparent molecular weights of marker proteins are indicated on the left.

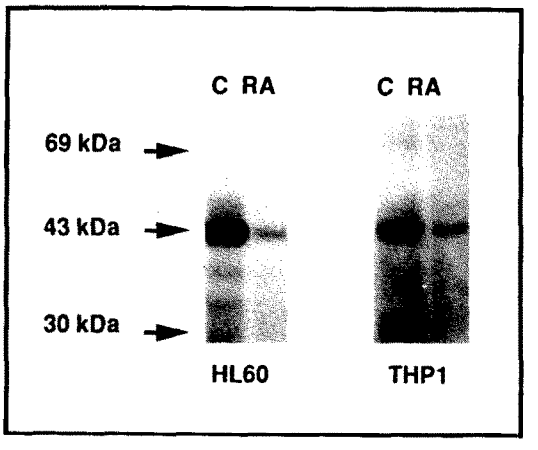

Fig. 3. The effect of RA-induced cell differentiation on the CTX-dependent ADP-ribosylation of membrane components of HL-60 and THP-1 cells. Membranes from untreated cells (C) and cells treated with RA for 5 days were incubated for 30 min at 32° with [α^{32}P]NAD and 25 μg/mL activated CTX, as described in Materials and Methods. Membranes from HL-60 and THP-1 were separated by SDS–12% PAGE, dried and autoradiographed overnight at −80°.

used as a mRNA quantitative marker. The GADPH mRNA levels were the same before and after treating cells with RA.

HL-60 and THP-1 cell membranes isolated from control and RA-treated cells were incubated with [^{32}P]NAD and CTX, as described in Materials and Methods. Figures 2 to 4 present autoradiographs of SDS polyacrylamide gel used to analyse ADP-ribosylation. Figure 2 presents autoradiographs of SDS polyacrylamide gel of HL60 membranes. The cells were pretreated for 16 hr with CTX (10^{-8} M, CTX pretreatment causes "down regulation" of Gsα) IAP (10^{-8} M), or nothing (C) and ADP-ribosylation was performed *in vitro* on membranes in the presence of CTX. CTX ADP-ribosylated one cell membrane protein of 44 kDa, thought to represent Gsα [10]. The fact that no labelling was obtained when cells were pretreated with CTX alone and no modification of the labelling occurred in cells pretreated by pertussis toxin corroborates this hypothesis. The same results were observed for THP-1 cells (data not shown).

Figure 3 shows that ADP-ribosylation of αs was highly diminished in membranes prepared from HL-60 and THP-1 cells after 5 days of RA treatment, when differentiation was maximal (2–8-fold). This modification was observed from 24 hr to 5 days, to the same extent, and was also observed with cell lysates (data not shown). However, there was a slight but not significant difference in 44 kDa band labelling in membranes prepared from 1.3% DMSO-treated cells (Fig. 4).

RA (10^{-5} M) had no effect when added directly to the ADP-ribosylation assay (data not shown).

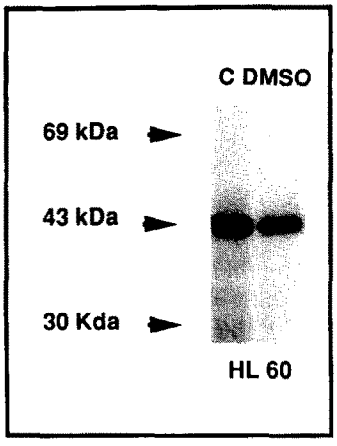

Fig. 4. The effect of DMSO-induced cell differentiation on the CTX-dependent ADP ribosylation of membrane components of HL-60. Membranes from untreated cells (C) and cells treated with DMSO for 5 days were incubated for 30 min at 32° with [α^{32}P]NAD and 25 μg/mL activated CTX as described in Materials and Methods. Membranes from HL-60 were separated by SDS–12% PAGE, dried and autoradiographed overnight at −80°.

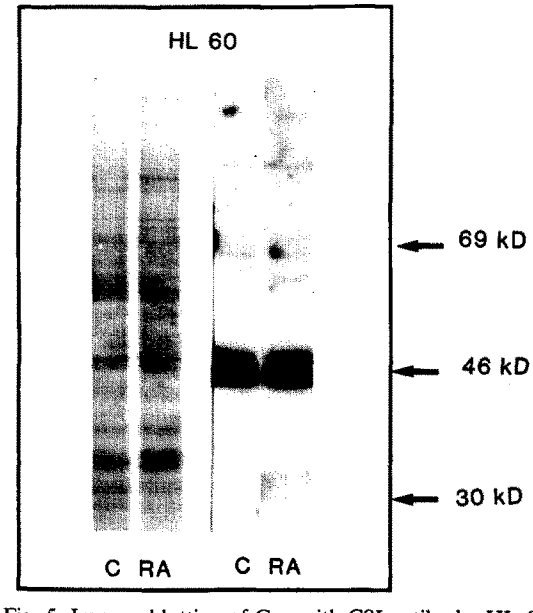

Fig. 5. Immunoblotting of Gsα with CSI antibody. HL-60 cells were treated for 5 days with 10^{-6} M RA. Cell membranes (50 μg per lane) were separated by SDS–10% PAGE, transferred onto nitrocellulose, immunoblotted with CSI antibody (1/300 final dilution), revealed by ^{125}I protein A and then monitored by autoradiography. The apparent molecular weights of marker proteins are indicated on the right. The coloured nitrocellulose (Ponceau red) is represented on the left. The autoradiogram (24 hr exposure) is from a single experiment and is representative of three separate experiments.

To confirm the change in the Gsα observed with CTX, membranes from control and RA-differentiated HL-60 and THP-1 cells were subjected to immuno-blotting using a polyclonal antisera specific for αs. The results obtained using this procedure are presented in Figs 5 and 6 and show that, in contrast to the results obtained with CTX-catalysed ADP-ribosylation, the same amount of protein was found before and after differentiation of both cell lines.

DISCUSSION

Since we had observed previously that the response of adenylate cyclase to known effectors is diminished after RA-induced differentiation in HL-60 and THP-1 cells, we examined the elements of the signal transduction pathway utilized by RA and, in particular, the stimulatory G-binding protein Gs (Ref. 8 and manuscript in preparation).

Our data show that RA did not modify the Gsα gene transcripts or the synthesis of the protein, suggesting a postranscriptional effect of RA.

Specific G-proteins act as substrates for ADP-ribosylation by CTX and IAP. The data presented in this study indicate that HL-60 and THP-1 membranes contain one Gsα protein with an apparent molecular weight of 44 kDa. In contrast with ADP-ribosylation where Gs was represented by one band of 44 kDa, the immunoblots with antiGsα showed three bands of 44, 46 and 48 kDa. Many of the antipeptide antisera so far produced demonstrate cross-reactivity with a range of other proteins besides the G-proteins but only Gsα is down regulated when the cells are pretreated by CTX [22]. The 44 kDa band disappeared when the cells were incubated for 24 hr with 10^{-8} M CTX before immunoblotting, suggesting that it corresponds to αs; the two other bands (46 and 48 kDa) were still present under these experimental conditions and were independent of CTX (data not shown).

In both cell lines, RA-induced differentiation was accompanied by a decrease in the CTX-catalysed ADP-ribosylation of Gsα. Moreover, the same decrease in Gsα ADP-ribosylation was not detected in cells induced to differentiate in the presence of 1.3% DMSO, indicating that it is the result of a specific action of RA rather than a consequence of the differentiation process.

A number of hypotheses can be put forward to explain the inhibitory effect of RA on CTX-catalysed ADP-ribosylation including: (1) a decreased availability of GTP; (2) retinoylation of Gsα at the site which is ADP-ribosylated by CTX, (3) loss of a membrane component which is necessary for the CTX-catalysed ADP-ribosylation; and (4) ADP-ribosylation of GSα by an endogenous mono-ADP-ribosyltransferase.

It has been published previously that RA and inhibitors of inosine monophosphate dehydrogenase, such as tiazofurin, which induced HL-60 cell differentiation, produce an early and marked depletion of the intracellular GTP pools in these cells [23, 24]. Also a decrease in CTX-catalysed ADP-ribosylation of GSα has been shown to occur

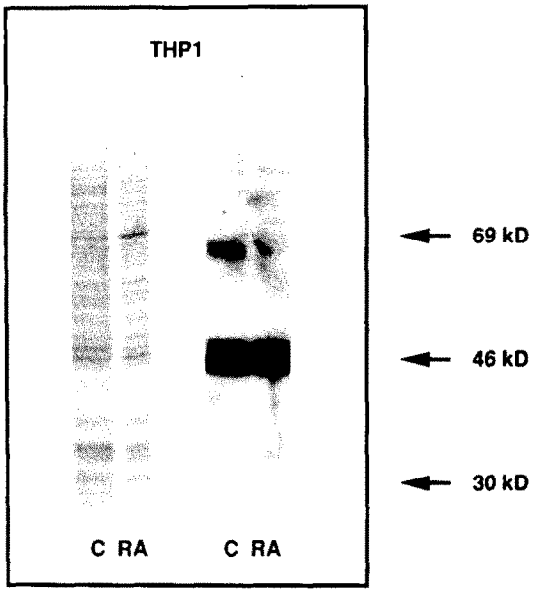

Fig. 6. Immunoblotting of Gsα with CSI antibody. THP-1 cells were treated for 5 days with 10^{-5} M RA. Cell membranes (50 μg per lane) were separated by SDS–10% PAGE, transferred onto nitrocellulose, immunoblotted with CSI antibody (1/300 final dilution), revealed by ^{125}I protein A and then monitored by autoradiography. The apparent molecular weights of marker proteins are indicated on the right. The coloured nitrocellulose (Ponceau red) is represented on the left. The autoradiogram (24 hr exposure) is from a single experiment and is representative of three separate experiments.

during the tiazofurin-induced differentiation of HL-60 [25]. However, in this latter case, a decreased ADP-ribosylation catalysed by PTX was also noted, an effect not seen with RA. Since the toxin-catalysed ADP-ribosylation of the cell membrane preparations has been performed by us as well as by Kharbanda *et al.* [25] in the presence of exogenously added GTP (100 μM), it is unlikely that the decrease in ADP-ribosylation of the band corresponding to Gsα was the result of a depletion of intracellular GTP pools.

Two conflicting results have been published on the covalent binding (retinoylation) of RA to proteins in HL-60 cells: Takahashi and Breitman [26] reported the almost exclusive retinoylation of a nuclear protein of M_r 55 kDa thought to be the RA nuclear receptor α; in contrast, Almagor and Bar-Tana [27] reported the retinoylation of only one membrane protein of M_r 25 kDa. In neither case was binding of radioactively labelled RA detected in proteins with a molecular weight corresponding to that of Gsα, although this does not constitute a clear proof that Gsα cannot be retinoylated.

Recently, Almagor and Bar-Tana [27] also showed that incubation of non-differentiated HL-60 cells with high specific activity myristic acid resulted in the specific myristoylation of a 25 kDa membrane protein, distinct from the one which would be retinoylated. This reaction was inhibited within 24 hr, after addition of RA to the incubation medium under conditions of RA-induced differentiation of the cells. However, differentiation induced by DMSO did not affect the myristoylation of the 25 kDa protein. It has been published that CTX-catalysed ADP-ribosylation of Gsα requires a protein cofactor ARF which is a G-protein of about 21 kDa, myristoylated at its amino terminus [28]. It is thus tempting to speculate on the identity of these two proteins and on the loss of anchorage of ARF to the plasma membrane during RA treatment of HL-60 and THP-1 cells, leading to the inhibition of CTX-catalysed ADP-ribosylation, as described in our study.

The existence of endogenous mono-ADP-ribosyltransferases in eukaryotic cells has been demonstrated [12]. Like microbial CTX and IAP, these enzymes catalyse the transfer of an ADP-ribose moiety of NAD to G-proteins. The RA effect could be the result of activation of an arginine-specific ADP-ribosyltransferase such as that characterized in turkey erythrocytes [29] or skeletal muscle membranes [30]. Thus, Morinaga *et al.* [31] have shown that HL-60 cell membranes contain at least two types of ADP-ribosyltransferases and it is probable that similar enzyme activities also exist in the cytosol fraction, as in turkey erythrocytes [32]. Stimulation of the activity of an endogenous arginine specific ADP-ribosyltransferase by RA would explain the decreased CTX-analysed ADP-ribosylation of Gsα as well as the increase in basal adenylate cyclase activity measured in HL-60 and THP-1 cell membranes (Ref. 8 and unpublished results). It would also explain the synergistic effect of CTX and RA in the differentiation of these cells [33].

We have shown previously that RA interferes with the activity of a G-protein Gp, which regulates the activity of phosphoinositide-specific phospholipase C [34]. The data presented in this study demonstrate that RA modifies the activity of another G-protein, Gs, which stimulates the activity of adenylate cyclase. This is in agreement with the data published recently by Galvin-Parton *et al.* [35] on the effect of RA on CTX-catalysed ADP-ribosylation of Gsα F9 teratocarcinoma cells. Associated with our already published data on the effects of RA on the modification of the activities of Na^+/K^+-ATPase and the Na^+/H^+ antiport [36], the data presented herein suggest that RA might act, at least in part, through modulation of the production of intracellular second messenger.

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