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U937 cells deprived of endogenous annexin 1 demonstrate an increased PLA_2 activity

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1 Annexin 1 (An 1), a phospholipid and calcium binding protein, is strongly expressed in differentiated U 937 cells. In attempting to correlate the expression of An 1 with phospholipase A_2 (PLA_2) activity, U 937 cells were stably transfected both with a Sense and Antisense cDNA for An 1. PLA_2 activity was measured by Flow cytometry analysis utilizing the bis-Bodipy-C_{11}-PC fluorescent probe.

2 U 937 cells stably transfected with the sense or antisense vectors were differentiated for 24 h with phorbol 12-myristate 13-acetate (PMA, 6 ng ml^{-1}). Both in undifferentiated and differentiated cells, the Antisense clone (36.4 AS) showed consistently higher PLA_2 activity than the control Sense clone (15 S).

3 Since the fluorescent probe measures the total PLA_2 activity, we used two different stimuli, PMA: (100 ng ml^{-1}) or lipopolysaccharide (LPS, 10 ng ml^{-1}), and two different inhibitors, to discriminate the PLA_2 involved (namely arachidonyl trifluoromethyl ketone or AACOCF3, which is specific for the cytosolic PLA_2, and SB 203347 specific for the secretory PLA_2).

4 In the Antisense clone the inhibitory effect of AACOCF was stronger [68%, $P<0.025$] than in the Sense, which may reflect the lower endogenous level of An 1 present in the cells. On the contrary, the inhibitory effect of SB 203347 [60% of inhibition] was identical in both clones.

5 Since $cPLA_2$ activity is correlated with its phosphorylation, Western and shift blot analysis were performed. They did not show any significative difference between the phosphorylated and non phosphorylated form of the enzyme in both the differentiated or not, Sense and Antisense clones. Furthermore the tyrosine phosphorylation analysis of An 1 showed that less than 10% of An 1 was phosphorylated irrespective of PMA presence or absence.

6 From the pattern of inhibition observed, we propose that the endogenous unphosphorylated form of An 1 may act intracellularly to block the activity of a cytosolic PLA_2.

Keywords: Annexin; cytosolic phospholipase A_2; secretory phospholipase A_2; inflammation

Introduction

Annexins or lipocortins comprise a large family of calcium-phospholipid-binding proteins. They are defined structurally by a conserved C-terminal region that contains four or eight repeating units of about 70 amino acids each. These conserved repeats account for the shared abilities of annexins to bind both calcium and phospholipids, whereas specific functions of each annexin are determined by their type-specific N-terminal regions.

Annexin 1 (An 1), the first identified member of the family has been shown to have some anti-inflammatory properties ranging from anti-edema (Cirino *et al*., 1989; Becherucci *et al*., 1993) to anti-migratory effect on polymorphonuclear cells. (Errasfa & Russo-Marie, 1989; Perretti & Flower, 1993) and/or anti-pyretic action (Davidson *et al*., 1991). These anti-inflammatory properties have been related to the ability of An 1 to inhibit phospholipase A_2 (PLA_2) activity *in vitro* systems as well as in cellular models (Hirata *et al*., 1982; Davidson *et al*., 1987; Errasfa & Russo-Marie, 1989; Comera *et al*., 1990; Russo-Marie, 1992).

Phospholipase A_2 (PLA_2) is thought to play a key role in inflammation by releasing arachidonic acid esterified at the sn-2 position of glycerophospholipids giving rise to the formation of the lipid inflammatory mediators: prostaglandins, leukotrienes and paf-acether (van den Bosch, 1980; Vadas *et al*., 1993). At the present day, different mammalian PLA_2s are known to be involved in the release of arachidonic acid. These PLA_2 enzymes belong to different types (Roberts, 1996; Dennis, 1997): the type II 14 kDa enzyme, known to exist both as an extracellular (Kramer *et al*., 1989) and cell-associated form (Marshall & Rshak, 1993), the cytosolic 85 kDa PLA_2 ($cPLA_2$) (Kramer *et al*., 1991) and the other cytosolic PLA_2, named $iPLA_2$ (for Ca^{2+}-independent PLA_2) (Balsinde *et al*., 1994). Although it is generally agreed that 85 kDa $cPLA_2$ is the best candidate for receptor-coupled arachidonic acid liberation, and $iPLA_2$ for replenishing the membrane with arachidonic acid for further release, the precise role of $sPLA_2$ is less clear. This 14 kDa protein has less preference for fatty acid in sn-2 position and therefore for arachidonic acid release. It is secreted by a number of cells in which its expression is either constitutive (platelets and neutrophils) or induced by inflammatory cytokines (chondrocytes, fibroblasts, macrophages) (Vadas *et al*., 1993). $sPLA_2$, which displays proinflammatory properties, is also detected in plasma of patients suffering from septic shock, strongly suggesting a possible involvement of this enzyme in the inflammatory reaction (Fourcade *et al*., 1995).

We have shown previously (Solito *et al*., 1991) that An 1 is strongly induced in PMA treated U 937 cells which become macrophage like cells expressing both CD 14 and CD11c markers (Solito *et al*., 1994). Using this model of differentiated cells as well as another model of epithelial cells in culture, we showed that An 1 is further induced and exported to the outer cell membrane in the presence of glucocorticoids (Solito *et al*.,

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1994) and of IL6, mimicking inflammatory stimuli (Solito *et al*., 1998a,b). By peptide mapping it has also been shown that the unique tyrosine residue that is phosphorylated by Epidermal Growth Factor (EGF) receptor kinase is located at the N terminus (Tyr^{21}) (Pepinsky, 1991). Phosphorylation of An 1 results in the release of cPLA2 activity, rendering this enzyme activated and therefore implicated in the regulation of prostaglandin-associated process (Skouteris & Schroder, 1996). Since An 1 is both increased in the intracellular compartment as well as at the cell surface, we analysed the relationships between An 1 and cytosolic PLA_2 and/or $sPLA_2$. To do this we used U 937 cells stably transfected with the Sense or Antisense cDNA of An 1. We report here the first analysis on live cells of PLA_2 activity and its modulation by endogenous unphosphorylated An 1.

Methods

Construction of vectors expressing partial Sense and Antisense annexin 1 RNA

A 476 bp 5′ fragment from the full-length human AN 1 cDNA obtained by reverse PCR from the mRNA from U 937 cells comprising 48 bp of the 5′ non-coding region, the ATG translation initiation codon and 409 bp of the coding sequence, was inserted at the *Bam*HI and *Hind*III sites of the pBluescript plasmid (pBlue 15 S). An Antisense clone with the inverted restriction site was called pBlue 36 AS. Standard procedures, as described by Sambrook *et al*. (1989) were used for DNA engineering. Both the Sense or Antisense constructs were further subcloned between the sites *Hind*III and *Not*I in the commercial vector pRC/Cytomegalovirus (CMV) (Invitrogen). The constructions were sequenced and monitored by restriction analysis.

Cell culture and transfection

U 937 cells maintained in culture with RPMI medium supplemented with 10% fetal calf serum at 37°C under a 5% CO_2 atmosphere were plated at 5×10^5/plate the day before the transfection, then cotransfected with the plasmid pRc/CMV containing the Sense or Antisense An 1 cDNA of 476 bp (Figure 1a), together with the plasmid containing the reporter gene Lac-Z, in dextran (1 mg ml^{-1}) TBS buffer as described (Ausubel *et al*., 1995).

Fluorescein di-β galactopyranoside (FDG) staining and cell sorting

Forty-eight hours after the transfection, cell sorting was performed as already described (Nolan, 1988; Russo-Marie, 1992). Briefly 10^7 cells per ml in staining medium containing 300 μM chloroquine were aliquoted into flow cytometric analysis (FACS) tubes and placed for 20 min in a 37°C water-bath. 100 μl of pre-warmed 2 mM fluorescein di-β-D-galactopyranoside (FDG by Molecular Probes) in H_2O were mixed and placed back at 37°C. The hypotonicity was maintained on for 60 s, and finally the FDG loading was stopped by adding 2 ml of ice-cold staining medium containing 300 μM chloroquine. FACS was performed on an Epics-Elite (Coultronics) Flow Cytometer equipped with an Argon ion laser beam operating at 488 nm using 15 mW of power to excite the fluorescein isothiocyanate (FITC) and propidium iodide. The data were stored in list mode using Elite Software 4.02. A gate was drawn selecting fluorescein-positive cells which were sorted onto 96-well plates using the autoclone II (50 cells/well). Cells that were found positive for LacZ expression had also retained the pRc/CMV vector. Those positive cells were sorted in order to obtain a minimal number of cells and put in selection with G 418. Cells were further maintained in a selection medium (G 418) for 2 weeks. Then each selected clone was analysed for An 1 expression both by Western blot and by FACS analysis as described below. Different Sense or Antisense clones were obtained after selection.

Western blot analysis

A total of 30 μg of total protein from U 937 clone 15 S or clone 36.4 AS were separated on 10% polyacrylamide gels according to the method of Laemmli (Laemmli, 1970) and electroblotted onto nitrocellulose membranes (BIORAD). Immunostaining was performed using a rabbit polyclonal antibody directed against the entire An 1 or cytosolic PLA_2 or secretory PLA_2 (expressed in a yeast expression vector as recombinant protein, Solito *et al*., unpublished results) or α-tubulin and the immunoreactive bands were detected using ECL (Amersham).

For immunoprecipitation analysis, 30 μg of protein were immunoprecipitated with a specific anti An 1 antibody directed against the N-terminal epitope. Immunostaining was performed using a monoclonal antibody directed against phosphotyrosine (p-Tyr), and the immunoreactive bands were detected as described before. Densitometric analysis was carried out using an Ultrascan XL Laser Densitometer (Agfa).

FACS analysis of the protein

Briefly, cells treated or not with PMA were fixed in 2% paraformaldehyde and incubated for 30 min at room temperature, then washed in 25 mM HEPES supplemented with 1 mM $CaCl_2$ and $MgCl_2$ and saponin (0.025% from *Saponaria* species). Non-specific binding was blocked with human IgG (1 mg ml^{-1}), and the sample further incubated with an antibody against An 1 (Solito *et al*., 1994). Log Fluorescence histograms (256 channels) were obtained from 5000 viable cells for each sample. Using the Elite 4.02 Data Analysis System from Coultronics, mean channel number fluorescence was used to assess differences in fluorescence intensity.

RNA analysis

U 937 cells clones were treated with 6 ng ml^{-1} PMA for 24 h then the RNA was extracted and hybridized as previously described (Ambrosetti *et al*., 1996). Following hybridization to the An 1 cDNA probe Antisense or Sense, filters were hybridized to a DNA fragment coding for glyceraldehyde-3-phosphate dehydrogenase GraP-DH in order to quantify the RNA loaded on the gels and compare the intensity of hybridization obtained in different lanes. RNA blot hybridization was carried out using standard protocols. RNA–DNA hybridization was quantified by densitometric computer analysis in a series 400 Phosphorimager from Molecular Dynamics.

Measure of PLA_2 activity

The technique described by Meshulam *et al*. (1992) was used with some slight modifications. Briefly, the fluorescent derivative, bis-BODIPY-C_{11}-PC (4,4-difluoro-5,7-dimethyl-4-

bora-3a,4a-diaza-s-indacene-3-undecanoyl-sn-glycero-3-phosphocholine by Molecular Probes) was combined with phosphatidylserine (PS, Sigma) at 1 : 9 molar ratio in ethanol and dried under nitrogen then dessicated overnight. The dried film of lipids was redissolved in PBS at 60 μg ml^{-1}, vortexed and sonicated for 30 min on ice as described (Meshulam *et al*., 1992). 6×10^7 cells per ml in PBS + 0.1% bovine serum albumin (BSA) were incubated for 15 min at 37°C with 2 volumes of bis-BODIPY-C_{11}-PC-labelled liposomes. Cells were washed three times with PBS then diluted 10 fold into PBS with Ca^{2+}/Mg^{2+}. FACS analysis of PLA_2 activity was performed with the Epics Elite cytofluorometer described previously, (excitation at 488 nm-argon ion laser, and detection through a 530 nm-centered bandpass filter). Data were collected for at least 5000 cells per sample. Briefly, when the probe is incorporated in cell membranes the proximity of bis-BODIPY-C_{11}-PC fluorophores on adjacent phospholipid acyl chains results in self-quenching of fluorescence, which is alleviated by release of a bis-BODIPY-labelled acyl chain in the presence of active phospholipase A_2.

In some cases, in order to validate the method described above, the release of ^{3}H-arachidonic acid from prelabelled cells was performed as already described (Comera *et al*., 1990). Cells were incubated overnight in the presence of 0.1 μCi ml^{-1} of ^{3}H-arachidonic acid in culture medium containing 10% FCS. After measuring the incorporation of labelled arachidonic acid, cells were washed three times with PBS with Ca^{2+}/Mg^{2+}. At the end of the incubation period, the reaction was stopped by adding a cold solution containing 5 mM ethylene glycol-tetraacetic acid (EGTA), 150 mM NaCl and 1% BSA-free fatty acid (FFA). The medium was removed after centrifugation and radioactivity was measured in a beta counter.

The respective importance of cytosolic PLA_2 and $sPLA_2$ was studied using selective inhibitors. Involvement of cytosolic PLA_2s (c- or i-PLA_2) was investigated by using arachidonyl trifluoromethyl ketone (AACOCF3), an analogue of arachidonate, this inhibitor displays a specificity for both cytosolic PLA_2s (c- or i-PLA_2) versus $sPLA_2$ (Trimble *et al*., 1993). For $sPLA_2$ we used a specific inhibitor SB 203347 described by Marshall *et al*. (1997). PLA_2 activity was measured in different conditions: (i) Untreated or PMA-induced cells: U 937 clones 15 S or 36.4 AS were pretreated for 24 h with or without 6 ng ml^{-1} PMA, then labelled with bis-Bodipy-C_{11}-PC cells and PLA_2 activity measured as described above; (ii) After stimulation: U 937 clones 15 S or 36.4 AS were pretreated for 24 h with 6 ng ml^{-1} PMA as just described above, then further incubated for 30 min with different concentrations of inhibitors: AACOCF3 or SB 203347 and further stimulated with PMA (100 ng ml^{-1}, 30 min) or lipopolysaccharide (LPS: 10 ng ml^{-1}, 2 h). Cells were then labelled with bis-Bodipy-PC cells and PLA_2 activity measured as described above.

Statistical analysis

Statistical analysis was performed using Student's *t*-test for unpaired samples. $P < 0.05$ is considered as significant.

Reagents

PMA, Saponin, fatty acid free BSA and all chemicals were purchased from Sigma Chemical Co. (Paris, France). The inhibitor arachidonyl trifluoromethyl ketone (AACOCF3) was from Calbiochem-Novabiochem Corporation (San Diego, CA, U.S.A.). The sPLA2 inhibitor SB 203347 was obtained by SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.) a gift of Dr L.A. Marshall. Bis-BODIPY- C_{11}-PC (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl-sn-glyero3phosphocholine) and the fluorescein di-β-D-galactopyranoside (FDG) was purchased from Molecular Probes (Leiden, The Netherlands). ^{3}H-arachidonic acid was purchased from NEN Life Science Products-France S.A. (Paris, France).

Antibodies

The α-tubulin monoclonal antibody was from Amersham and was used at a 1 : 1000 final dilution (Buckinghamshire, U.K.). Monoclonal antibody against An 1 was a gift of Dr J. Browning (Biogen, Cambridge, MA, U.S.A.) and was used at the final dilution 1 : 1000. The rabbit polyclonal antibody directed against the N-terminal epitope of the An 1 protein (Becherucci *et al*., 1993) was IgG purified and used at a final dilution 1 : 1000. The anti sPLA2 (1 : 5000) was prepared in our laboratory using recombinant sPLA2 obtained in yeast (Solito *et al*., unpublished results). The rabbit polyclonal antibody (1 : 1000) directed against An 1 was prepared in our laboratory using recombinant An 1 (*E. Coli* expressed). The monoclonal anti-phosphotyrosine and anti-cPLA2 antibodies were from Santa Cruz-Biotechnology (Santa Cruz, CA, U.S.A.). They were used respectively at a dilution of 1 : 400 and 1 : 1000, respectively.

Results

Stable transfection of annexin 1 Antisense RNA in U 937 cell line decreases annexin 1 expression

Figure 1B shows an histogram of expression of An 1 (measured by Western blot) in the Antisense clones (36.2, 36.4, 36.6) compared with the Sense clone 15 S and non transfected cells (U 937 control). Results indicate that the clone 36.4 AS showed a reduction of the An 1 expression of approximately 61% in unstimulated cells and 83% in PMA stimulated cells compared to the Sense clone (U 937 15 S). On the basis of the results obtained with the An 1 protein analysis we decided to further continue our study comparing the clone 15 Sense (15 S) and the clone 36.4 Antisense (36.4 AS). Figure 2A reports a Northern blot analysis of the Sense or Antisense clones treated or not with 6 ng ml^{-1} PMA for 24 h. PMA induced a strong expression of the mRNA in the Sense clone (as already found in the control cells) (Solito *et al*., 1991) while in the Antisense clone, the expression is significantly decreased. The expression of the protein was further analysed using FACS and this assay confirmed that the clone 36.4 AS (PMA treated or not) contains a reduced expression of the protein: compare histograms 3 (clone 36.4 AS) versus histograms 2 (clone 15 S) in Figure 2B.

Phospholipase A_2 activity in normal and An 1 deficient cells

The FACS profile of the phospholipase activity assay is shown in Figure 3A. Histograms 1 and 2 depict one typical experiment with the cells (clone 15 S and 36.4 as respectively) incubated with bis-BODIPY-PC and maintained in culture for 2 h in medium completed with 2% FCS. Histograms 3 and 4 (clone 15 S versus 36.4 AS) represent one typical experiment performed with the cells stimulated for 24 h with PMA then labelled with bis-BODIPY-C_{11}-PC and further incubated in complete medium (2% FCS). The intensity of fluorescence indicates the activation of PLA_2 which cleaves the BODIPY

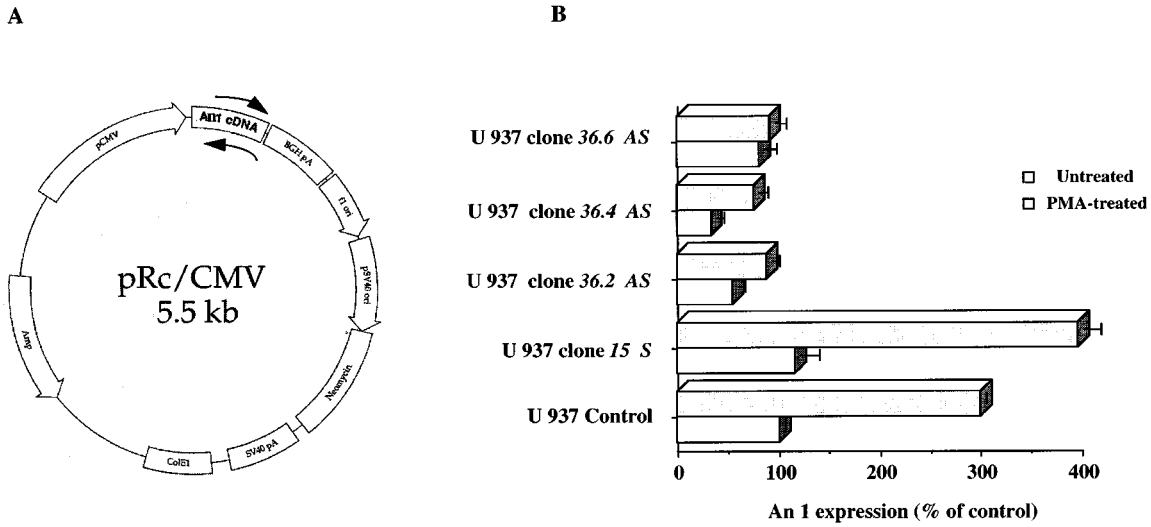

Figure 1 Down regulation of Annexin 1 (An 1) expression in U 937 clones transfected with the An 1 Antisense expression vector pRc/CMV. (A) Structure of the vector pRcCMV 5.5. (B) Densitometric analysis of Western blot of An 1 expression of cell extracts from parental not transfected U 937 cells (control) and from the clone 15 Sense or 36.2, 36.4 and 36.6 Antisense. Equal amount (30 μg) of total cell protein extracts from cells treated or not with PMA, were separated by electrophoresis and blotted onto nitrocellulose. The clones AS showed a significative decrease of An 1 expression compared to the clone 15 S or the Control. Each value was normalized on the internal control obtained with the immunodetection of α-Tubulin. Values are expressed as per cent of Control cells (U 937 not transfected), $n=3$.

group and causes an increase in fluorescence emission. Results of a series of experiments performed in both the Sense or Antisense clones are reported in Figure 3B. Antisense clone (36.4 AS) shows a higher PLA_2 activity as compared to the Sense clone (15 S).

In parallel, and in the same clones, PLA_2 activity was measured using the most classical technique measuring the release of ^{3}H-arachidonic acid from prelabelled cells (Table 1). In the Sense clone (15 S) the release of ^{3}H arachidonic acid was of 300% (as compared to non stimulated cells) whereas it was of 800% in the Antisense clone (36.4). These data perfectly overlap with our analysis of PLA_2 activity measured using bis-BODIPY-C11-PC and FACS analysis, eliminating the enzymatic involvment of a lysophospholipase or a PLA_1.

Western blot analysis of cytosolic and secretory PLA_2

As mentioned above, PLA_2 activity found in cells is associated at least with two different enzymes, the cytosolic PLA_2 (85 kDa) and the secretory PLA_2 (14 kDa). The presence of the two enzymes was analysed by Western blotting, in our clones, before and after treatment with PMA (Figure 4). In the experimental conditions used, no differences in the level of expression of both PLA_2s in the Sense and Antisense clones were observed. More, since $cPLA_2$ is present in two different forms, a phosphorylated active one (upper band) and a non phosphorylated one (lower band) we calculated the percentage of the phosphorylated enzyme on the total amount of the protein. Figure 4A shows that $cPLA_2$ phosphorylation was not different in our clones, before and after treatment with PMA.

Role of An 1 on cytosolic or secretory phospholipase A_2

Since the capability of endogenous An 1 to inhibit the cPLA2 has been reported to be correlated with its degree of tyrosine 21 phosphorylation (Skouteris & Schroder, 1996), we first analysed the pool of endogenous tyrosine phosphorylated An 1 in both the sense (15 S) or antisense (36.4 AS) clones. Figure 5 reports the densitometric analysis of the band, phosphorylated or not, at Western blot level. The not phosphorylated pool was strongly present compared to the phosphorylated band in both the Sense (15 S) or Antisense (clone 36.4 AS) clones.

Figure 6 shows the data obtained in clones 15 S and 36.4 AS in the presence of AACOCF3 and SB 203347. In a first series of experiments, (upper panels) cells were pretreated for 24 h with 6 ng ml^{-1} PMA, labelled with bis-BODIPY-C_{11}-PC and further incubated for 30 min with PMA (100 ng ml^{-1}). Inhibition by AACOCF3 (at the 0.3 and 3 μM concentrations; Figure 6A: upper panel) of the PLA_2 activity was significantly higher in the antisense clone than in the sense clone while the inhibition by SB 203347 (Figure 6B, upper panel) was identical, at all the concentrations tested in both clones. In another set of experiments, (Figure 6: lower panels) cells were pretreated for 24 h with PMA (6 ng ml^{-1}), labelled with bis-BODIPY-C_{11}-PC and further indubated for 2 h with LPS (10 ng ml^{-1}). The same profiles of inhibition as with PMA were found, although differences observed with AACOCF3 were not significant (see Figure 6A, B, lower panel).

Discussion

One of the underlying motifs of early research in the Annexin 1 field was the concept that its induction by glucocorticoids reflected an anti-inflammatory action that was directed at blocking phospholipase A_2 activity.

Since the early work on An 1, the PLA_2 field has rapidly advanced. The 14 kD secretory PLA_2 (sPA_2) has been implicated in eicosanoid release and inflammation. An 85 kD cytosolic form ($cPLA_2$) that lacks homology to $sPLA_2$ or to the pancreatic enzyme has been distinguished and isolated. $cPLA_2$ is inducible by cytokines and is activated by mitogenic and other factors through a complex signalling system involving MAP kinase (Qui & Leslie, 1994). The 85 kD $cPLA_2$ differs in

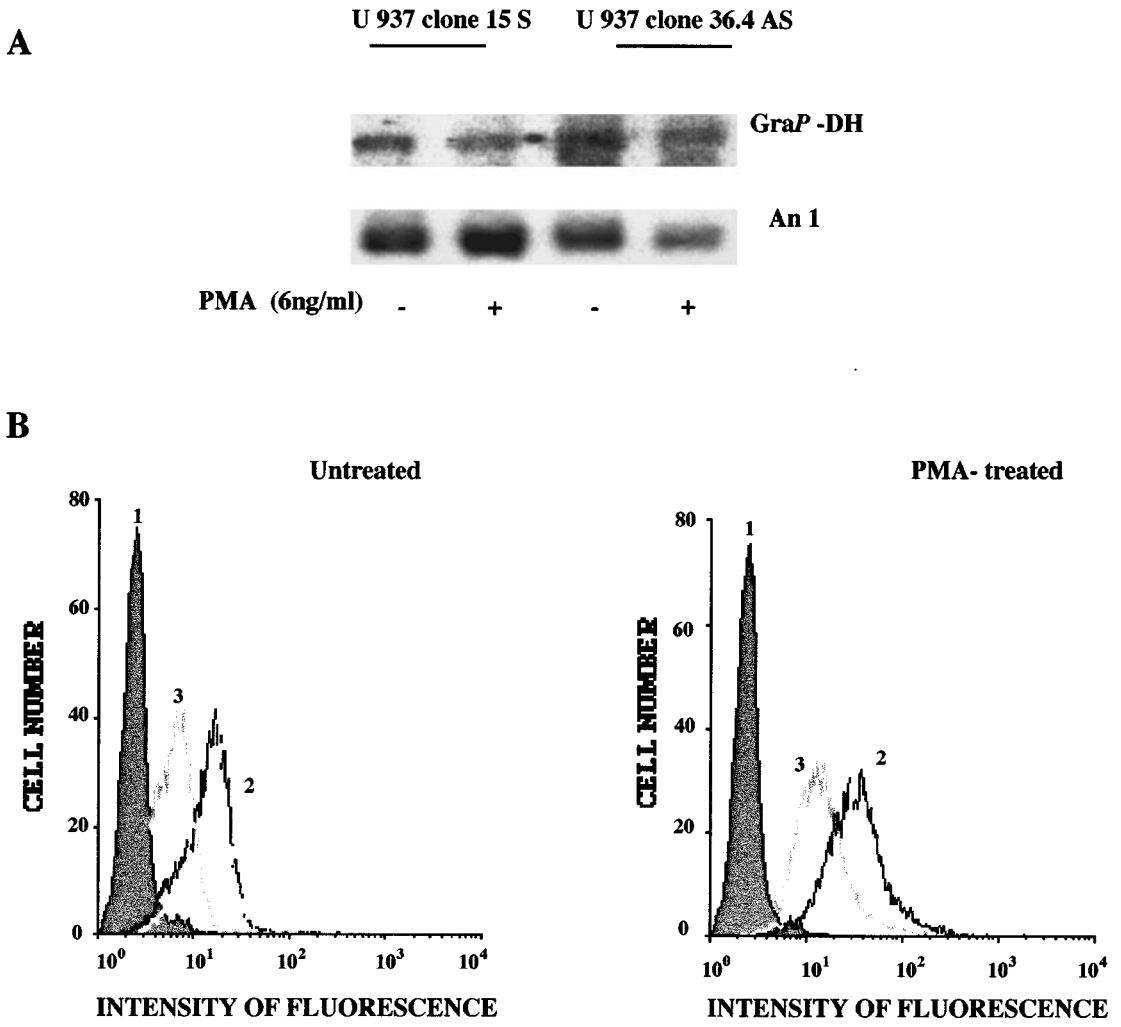

Figure 2 Northern, and FACS analysis of the clone Sense and Antisense, treated with PMA. (A) Northern blot analysis of RNA from U 937 cells transfected with the S or AS An 1 cDNA. Glyceraldehyde-3-phosphate dehydrogenase (GraP-DH) mRNA hybridization was used to compare the total amount of RNA between the different treated cells. One representative experiment of four different experiments with similar results is shown. (B) FACS analysis of An 1 expression. U 937, PMA treated or untreated. Fluorescence intensity (log 10 scale) is plotted against cell number. Histogram 1 represents the cells incubated with the second antibody alone (right or left panel). Histograms 2 are the cells from the clone 15 S in presence of the An 1 antibody (2). Histograms 3 represent the An 1 level in cells from the clone 36.4 AS (3).

almost every way from $sPLA_2$ and has preference for arachidonic acid implicating it also in the production of inflammatory mediators. More recently, another cytoplasmic PLA_2 has been identified ($iPLA_2$). This PLA_2 remodels membranes to allow arachidonic acid to be placed in the proper position in phospolipids for stimulated release (Dennis, 1997). Therefore, the inhibitory effects of An 1 on PLA_2 activity may cover different PLA_2s.

We and others have previously shown that An 1 was able to inhibit group I and II of phospholipases in various *in vitro* systems (Russo-Marie, 1992). To date, there have been few attempts to evaluate the action of An 1 on other PLA_2s, particularly in intact cells. Based on coprecipitation studies, one group has suggested that An 1 interacts directly with the 85 kD enzyme (Kim *et al*., 1994). In the A549 human lung adenocarcinoma cell line it has been reported (Croxtall *et al*., 1995) that the release of arachidonic acid is mainly catalyzed by the 85 kDa $cPLA_2$. Addition of glucocorticoids to A549 cells results in complete inhibition of PGE_2 release and subsequent growth arrest, suggesting that the induced increase of An 1 is inhibiting cPLA2 activity (Croxtall *et al*., 1996). Taken altogether, these data suggest that An 1 may also inhibit $cPLA_2$.

In order to address this question, U 937 cells were stably transfected with a sense and antisense cDNA for An 1. A clone was obtained with a significantly lower expression of An 1. As expected, this difference in the expression of the protein was exacerbated in PMA-differentiated cells (Solito *et al*., 1991; 1994).

A new technique was used to measure PLA_2 activity using FACS analysis that allows statistics on a limited number of living cells. The use of fluorescent lipids in investigations of cellular metabolism is already known. Sleight & Pagano (1984) reported metabolic processing of a 1-acyl-2 (N-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylcholine (C6-NBD-PC) in fibroblasts as an indicator that a fluorescent group does not interfere with substrate recognition by cellular phospholipases. Meshulam utilizing the same concept studied the activation of phospholipases in neutrophils by various agonists (Meshulam *et al*., 1992).

Using the same experimental approach, we report here that total PLA_2 activity is higher in clone 36.4 AS (where An 1 is

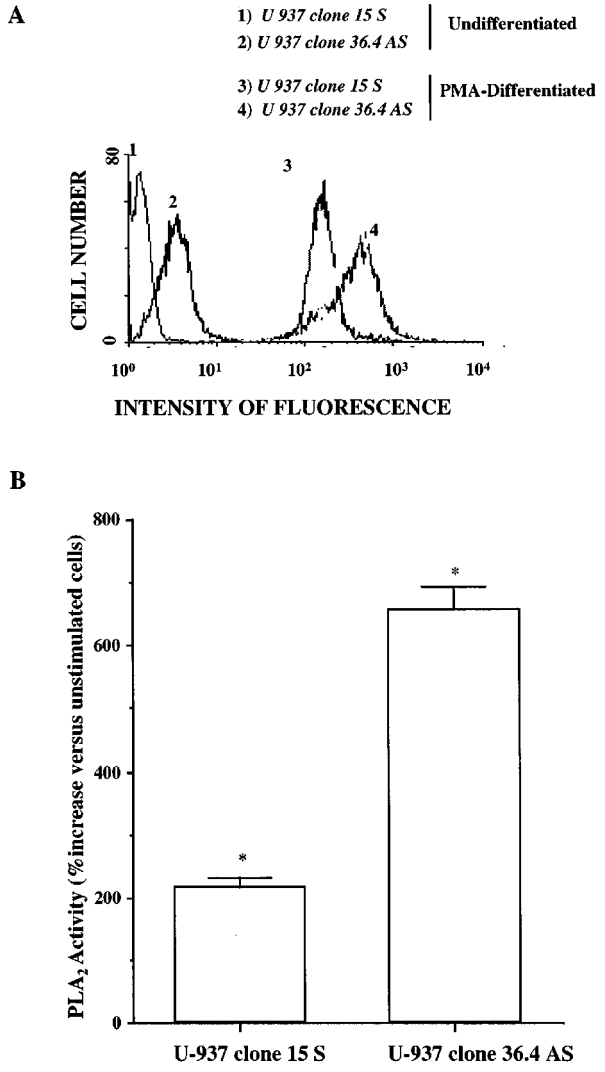

Figure 3 PLA_2 activity in the clone S or AS. (A) Total phospholipase A_2 activity was measured, in the clones transfected with the An 1 Sense or Antisense cDNA at FACS. The histograms are represented in intensity of fluorescence versus number of cells. The histograms 1 and 2 represent the cells (clone 15 S and 36.4 AS respectively) kept in culture and incubated with the bis-Bodipy-PC. The histograms 3 and 4 represent the cells treated with PMA and then incubated with the fluoro molecule. This is one representative pattern of six different experiments. (B) Representative data indicating the different phospholipase A_2 activity of the clone 15 S and 36.4 AS. The values are expressed as % increase in treated cells versus untreated cells (intensity of fluorescence). Columns represent mean (with s.e.m. showed in vertical bars) of one experiment performed in triplicate. Three other experiments gave similar results. $P<0.0005$ is referred to the clone *15 S* PMA treated versus the relative untreaded control, or the clone *36.4 AS* PMA-treated versus the relative untreated control.

Table 1 Effect of PMA on ^3H AA release by U 937 clone *15 S* and 36.4 *AS*

Sample	*^3H AA Release* (% increase versus unstimulated cells)
U 937 clone 15 S	289 ± 10
U 937 clone 36.4 AS	860 ± 37*

Measurement of ^3H arachidonic acid release from U 937 clone S or 36.4 AS with or without treatment with 6 ng ml^{-1} PMA for 24 h. Values are mean $\pm$ s.e.m. of two independent experiments performed in triplicate ($n=3$). $P<0.05$ clone 36.4 AS versus clone 15 S (Student's *t*-test).

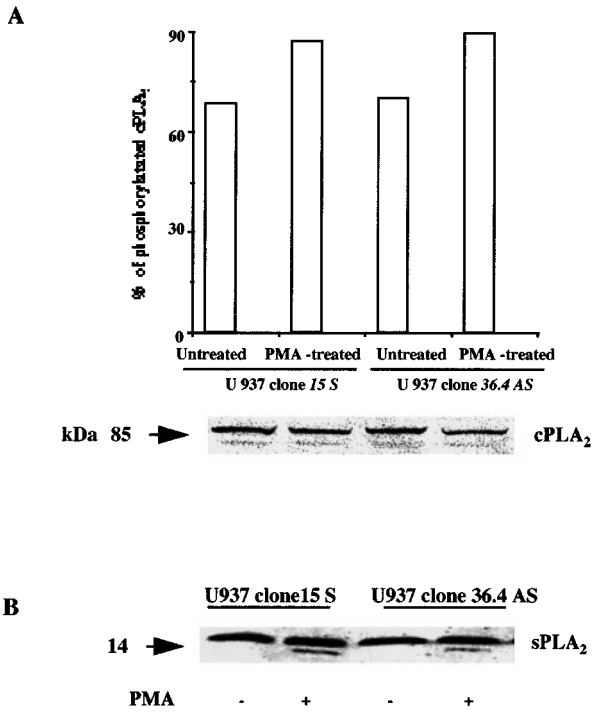

Figure 4 Immunoblot analysis of the 85 kDa and 14 kDa Phospholipases A_2. U 937 clones 15 S or 36.4 AS were treated or not with PMA. Total cellular proteins were extracted and analysed by SDS–PAGE. Immunoblots were performed with a specific antibody for 85 kDa PLA_2 or the 14 kDa secretory one. (A) the histogram represents the % of phosphorylated $cPLA_2$ on the total expression protein.

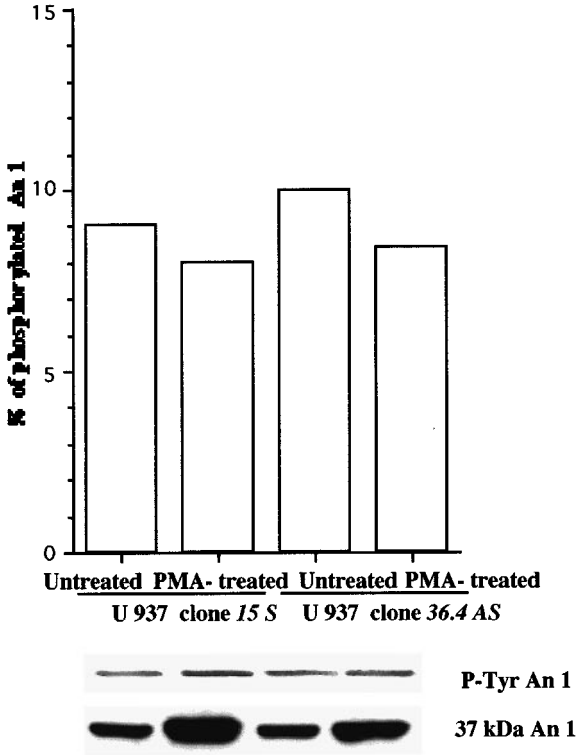

Figure 5 An 1 phosphorylation analysis. An 1 immunoprecipitated from lysates of cells treated or not with PMA, was probed with a P-Tyr antibody and after stripping with the anti-annexin 1. At the right side of the blot, molecular masses and names of the reacting species are shown. The % of phosphorylated An 1 was calculated on the total An 1 bands (phosphorylated and not). The data are representative of two independent experiments.

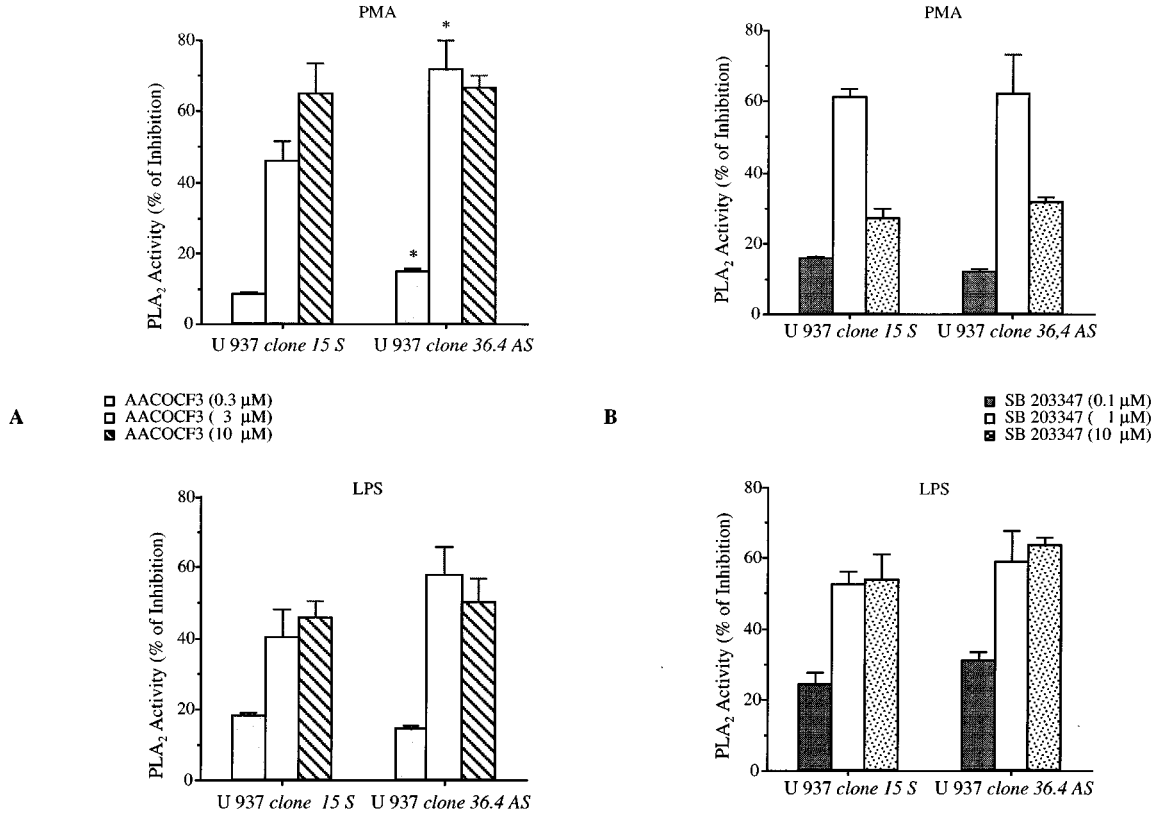

Figure 6 The effect of AACOCF3 and SB 203347 on stimulated U 937 clone S or AS. PLA_2 activity is expressed as % of inhibition (as % of intensity of fluorescence). Data represent mean ± s.e.m, $n=3$, of three different experiments. Each value is significantly different from every relative control. *indicates significantly different versus the clone 15S at $P<0.025$ (Student's *t*-test).

decreased) than in the clone 15 S (where An 1 level is normal) suggesting a role of An 1 in inhibiting PLA_2 activity. Since the fluorescent probe measures total PLA_2 activity and cannot discriminate between the different groups of PLA_2 two different inhibitors were used for this purpose. Decreased PLA_2 activity, which may be either $cPLA_2$ or $iPLA_2$ was observed in the presence of the inhibitor AACOCF3 in differentiated cells further treated with PMA or with LPS. The $sPLA_2$ inhibitor, SB 203347, described by Marshall *et al.* (1997) induced also a decreased PLA_2 activity in the same conditions. These results suggest that in our experimental conditions, the PLA_2 activity measured is due to the activation of both cytosolic PLA_2 and $sPLA_2$ suggesting a possible connection between both PLA_2 pathways of activation (Balsinde & Dennis, 1996). In the antisense clone the inhibitory effect of AACOCF3 was stronger than in the Sense clone, whereas no difference was observed in the inhibitory effect of SB 203347 in the same experimental conditions. These data suggest that only a cytoplasmic PLA_2 is affected by the deprivation of endogenous An 1, and that in our conditions, endogenous An 1 does not regulate $sPLA_2$ activity. The significant increase in PLA_2 inhibition in the antisense clone, as compared to the sense clone after AACOCF3 treatment, may reflect the lower level of An 1 present in the cell, suggesting that at intermediate concentrations such as 0.3 and 3 μM, the apparent affinity of AACOCF3 for PLA_2 is significantly higher. However, our results do not allow to discriminate between the two main types of cytosolic PLA_2 namely $cPLA_2$ and $iPLA_2$.

Although our data seem rather straightforward, a simple inhibitory role of annexin 1 on $cPLA_2$ can be questioned. Indeed, two recent papers, based on the use of antisense technology for analysing the role of An 1 report an opposite function for this protein. Skouteris & Schröder (1996) showed, in A 549 cells stimulated with the hepatocyte growth factor (HGF), that the decrease of endogenous An 1 inhibit both cell proliferation and the production of PGE_2. Their data suggest that tyrosine phosphorylation of An 1 induced by HGF is responsible of an augmented $cPLA_2$ activity. Similarly, Hayashi *et al.* (1993) showed that TEA3A1 thymic epithelial cells, transfected with antisense An 1 cDNA, have a significantly lower PGE_2 production. In sense An 1 transfected TEA3A1 cells, the PGE_2 release was increased and accompanied by higher levels of $cPLA_2$ activity. Taken together, these two papers suggest that PLA_2 activity may be regulated positively by An 1.

Although our data seem in contradiction with these reports, they can shed new light on the endogenous role of An 1. In our experiments, we used a promonocytic cell line in which PMA induces differentiation together with a growth arrest after 24 h. We did not find any significant difference between the Sense or Antisense clones in term of CD14/CD11c expression compared to the U 937 non transfected cells as already reported (Solito *et al.*, 1994). This effect is correlated with an increased expression of An 1 (Solito *et al.*, 1991; 1994), without any significant up-regulation of the phosphorylation on Tyr-21. To interpret these contradictory data, we suggest that An 1 exists *in vivo* under two (unphosphorylated and tyrosine phosphorylated) forms.

When An 1 is present in the cells under its unphosphorylated form, it may inhibit $cPLA_2$ by competing for its substrate. A diminution of An 1 in these conditions would lead

to an increased $cPLA_2$ activity. During cell proliferation and upon tyrosine phosphorylation induced by growth factors such as EGF or HGF, tyrosine phosphorylated An 1 may change its affinity for phospholipids and relieve its inhibitory effect on $cPLA_2$ (Hirata *et al*., 1984). A diminished An 1 in these conditions would lead to a diminished $cPLA_2$ activity (Skouteris & Schroder, 1996).

In conclusion the present work tries to delineate a role for cellular An 1 as an endogenous inhibitor of PLA_2. From the pattern of inhibition observed, we propose that endogenous An 1 acts intracellularly to regulate a cytosolic PLA_2, a physiological mechanism that could be related with the control of cell growth (Croxtall *et al*., 1995; Hayashi *et al*., 1993; Skouteris & Schroder, 1996). The mechanism involved in this inhibition (direct interaction or substrate depletion) is unknown at the present time.

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