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GDP-RIBOSYL CYCLASE ACTIVITY AS A MEASURE OF CD38 INDUCTION BY RETINOIC ACID IN HL-60 CELLS

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Retinoic acid (RA) treatment of HL-60 cells induces surface expression of CD38. This lymphocytic antigen is also a novel bifunctional enzyme catalyzing the synthesis and hydrolysis of cyclic ADP-ribose (cADPR), a Ca^{2+} mobilizing metabolite of NAD^+. The synthetic activity of CD38 is very difficult to detect because of the concurrent hydrolytic activity. In this study, a Ca^{2+} release assay capable of detecting submicromolar concentrations of cADPR was used to demonstrate the induction of ADP-ribosyl cyclase activity in HL-60 cells by RA. Concommitantly, cADPR hydrolase activity was also increased. The results were further substantiated by using a newly developed assay for GDP-ribosyl cyclase activity. This assay uses NGD^+ as substrate instead of NAD^+. The resulting fluorescent product, cyclic GDP-ribose, is resistant to hydrolysis and accumulates, making it a highly sensitive and convenient assay for CD38-like enzymes.

Intracellular Ca^{2+} release has long been recognized as an integral part of cell regulation. The two leading intracellular constituents which have been recognized for their Ca^{2+} release activities are inositol trisphosphate (reviewed in #1) and cADPR (reviewed in #2). Accumulating evidence indicates cADPR may be a regulator of the Ca^{2+}-induced Ca^{2+} release mechanism (3-5). Recently, the possibility that cADPR is involved in the signaling function of nitric oxide has been proposed (6).

The first indication that the lymphocytic antigen, CD38, may be involved in the metabolism of cADPR was suggested by the similarity of its amino acid sequence to that of the *Aplysia* ADP-ribosyl cyclase, a synthetic enyzme of cADPR (7). Subsequent work demonstrates that CD38 is a bifunctional enzyme with both ADP-ribosyl cyclase and cADPR hydrolase activities (8-11). The concurrent presence of the hydrolytic activity makes it very difficult to measure the cyclase activity of this type of enzyme. Indeed, several reports failed to detect the cyclase activity in a promyelocytic leukemic cell line, HL-60, following RA treatment, even though CD38 expression was induced (12-14).

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HL-60 cells are myeloid leukemia cells which can be induced to differentiate into either the monocytic or the granulocytic pathway. RA induced differentiation of these cells is associated with CD38 expression (15). Similar specificity is seen in lymphocytes. Only the early and late stage of B- and T-cells, but not the intermediate stages, express CD38 (reviewed in #16). Recent evidence shows that CD38 expression may also be involved in preventing apoptosis of human germinal center B cells (17). The fact that cADPR is a signaling molecule suggests that the enzymatic activity of CD38 may be important for its biological function.

Two different assays are used in this study to demonstrate that the induction of CD38 expression in HL-60 cells is associated with an increase in ADP-ribosyl cyclase activity. The first is a Ca^{+2} release bioassay, which is capable of detecting submicromolar concentrations of cADPR (4). The second assay measures the GDP-ribosyl cyclase activity. This novel assay utilizes NGD^+ instead of NAD^+ as a substrate for CD38. The product synthesized, cyclic GDP-ribose (cGDPR), is the guanine nucleotide equivalent to cADPR. In contrast to cADPR, cGDPR is resistant to hydrolysis by CD38 and accumulates (18). It is also fluorescent and allows continuous monitoring of the reaction fluorimetrically. The high sensitivity and convenience of this novel assay makes it the assay of choice for monitoring enzymes with ADP-ribosyl cyclase activity.

METHODS

Treatment of HL-60 cells. HL-60 cells were grown in culture as previously described (19) and treated with RA (0.1 μM) for 18 h or left untreated (control). After the treatment, the cells (2.7 to 10 x 10^7) were washed twice with phosphate buffered saline, pH 7.4, and sedimented by centrifugation at 1,500 rpm. The cell pellets were resuspended in 5 ml of 10 mM Tris, pH 7.4 containing 250 mM sucrose and 0.1 mM PMSF. The cells were lysed in this buffer by 4 freeze/thaw cycles and separated from lysate by centrifugation at 4,000 rpm for 10 min. The supernatant from the low speed spin was recovered and centrifuged at 100,000 x g for 1 hr to sediment the membranes. The pellets were resuspended in 10 mM Tris pH 7.4 containing 0.1 mM PMSF. Both membranes and supernatants from the high speed spin were examined for enzyme activities.

Ca^{2+} release assay for ADP-ribosyl cyclase. Ca^{2+} release was measured in homogenates of eggs from the sea urchin *Strongylocentrotus purpuratus* as described previously (4). The incubations of NAD^+ with HL-60 extracts were started with the addition of 1 mM NAD^+ and maintained at 37°. At various times, small aliqouts (1-2 μl) of the mixture were added to 0.2 ml of egg homogenates and the resulting Ca^{2+} release activity was compared with calibration curves generated with known concentrations of cADPR.

Fluorometric assay for GDP-ribosyl cyclase. The activity of ADP-ribosyl cyclase can be measured by using NGD^+ as substrate and following the production of cGDPR by the increase in fluorescence (18). Membranes of HL-60 cells (28 to 112 μg/ml protein) were incubated with 40 μM NGD^+ in 0.8 ml of 20 mM Tris pH 7.4 at 37°. The excitation wavelength was set at 300 nm and the emission wavelength was 410 nm. The amount of cGDPR produced was determined by comparing the fluorescence intensity with that of cGDPR standards.

HLPC assay. The nucleotides NAD^+, cADPR, and ADP-ribose, NGD^+, cGDPR and GDP-ribose are easily separated by HPLC on the anion exchange resin AG MP-1 using a non-linear gradient of trifluroacetic acid for elution as described previously (18). The amounts of various nucleotides were determined by comparing the areas of peaks from the HPLC elution

profiles with standards. NGD^+ was purchased from Sigma (St. Louis, MO) and further purified by the same HPLC procedure.

RESULTS AND DISCUSSION

Membranes from HL-60 cells treated with retinoic acid were incubated with NAD^+. At the start of the incubation, a small aliqout was added to the egg homogenates and no Ca^{2+} release activity was detected (trace labeled 0 in Fig. 1a). The activity increased as incubation progressed. In comparison, no increase in Ca^{2+} release activity was seen with membanes from control cells even after 50 min of incubation (Fig. 1b). The Ca^{2+} release by the incubation mixture can be blocked by 8-amino-cADPR (data not shown), a specific antagonist of cADPR (20), indicating it was due to production of cADPR during incubation.

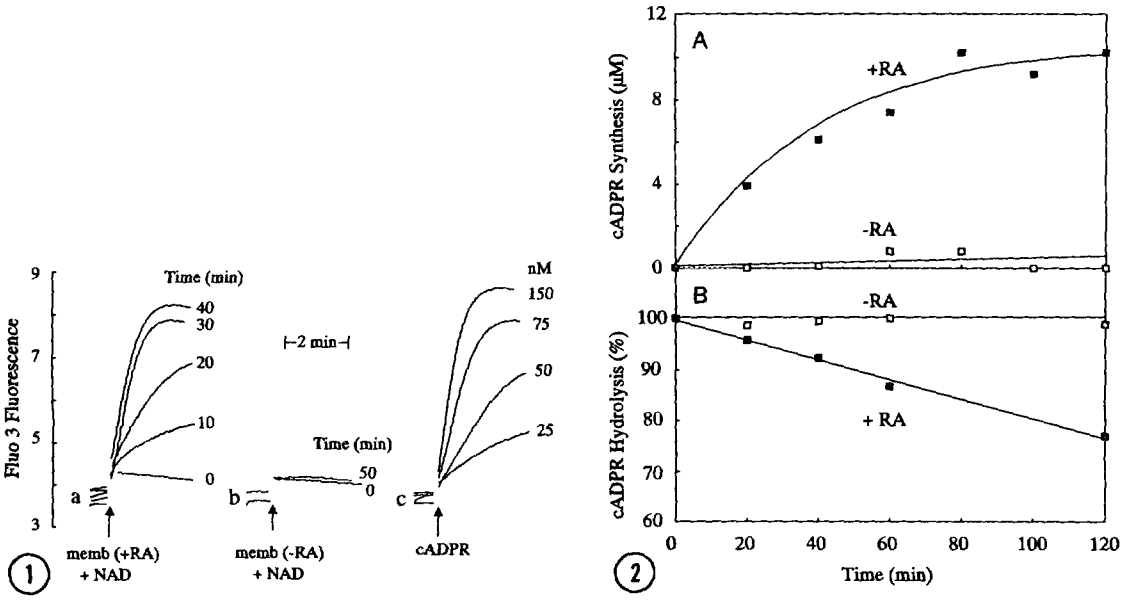


Figure 1. Ca^{2+} release assay of ADP-ribosyl cyclase activity. Membranes from RA treated (a, (+) RA, 0.43 mg protein/ml) and untreated HL-60 cells (b, (-)RA, 0.53 mg protein/ml) were incubated with 1 mM NAD^+ at 37° C and aliquots were diluted 100-fold into sea urchin egg homogenates at the times indicated. The resulting Ca^{2+} release was measured by Fluo 3 and compared to calibration curves constructed using authentic cADPR (c).

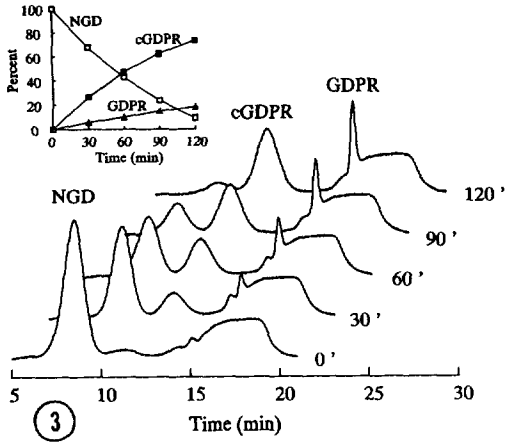
Figure 2. Time courses of the synthesis and hydrolysis of cADPR. A. Production of cADPR catalyzed by membranes from RA treated (+RA) and control (-RA) HL-60 cells was measured using the Ca^{2+} assay as described in Fig. 1. B. Membranes from RA treated ((+) RA, 0.32 mg protein/ml) and untreated HL-60 cells ((-)RA, 0.4 mg protein/ml) were incubated with 1 mM cADPR at 37°C and 20 μl aliquots were analyzed by HPLC at the times indicated.

Comparison of the Ca^{2+} release activity with that produced by authentic cADPR (Fig. 1c) allowed for quantification of cADPR production. As shown in Fig. 2A, membranes from control cells did not catalyze detectable cADPR synthesis even after 100 min of incubation. The production of cADPR catalyzed by membranes from RA treated cells increased for the first 40 min and then leveled off. From the linear portion of the curve, the rate of cADPR production was determined to be 0.563 nmol/mg protein/min. The leveling-off of cADPR production indicated a steady state was reached and suggested the presence of cADPR hydrolysis activity in the membranes. This was found to be case as shown in Fig. 2B. Membranes from RA treated cells, but not those from control cells, catalyzed the hydrolysis of cADPR. The rate of cADPR hydrolysis measured with 1 mM substrate was 6.06 nmol/mg protein/min. The presence of both ADP-ribosyl cyclase and cADPR hydrolase activities in membranes from retinoic acid treated cells is consistent with RA inducing the expression of CD38, which has been shown to be a bifunctional enzyme (8-11).

The difficulty in detecting ADP-ribosyl cyclase activity is clear from the results shown in Fig. 1 and 2. Using 1 mM NAD^+ as substrate only about 10 μM cADPR (1%) was produced and the rest was eventually hydrolyzed to ADP-ribose. Without the highly sensitive Ca^{2+} release assay for cADPR, the cyclase activity can be easily missed (12-14). A new assay has recently been developed to alleviate this difficulty. The assay employs NGD^+ as an alternative substrate (18). Membranes from RA treated cells were incubated with NGD^+ and aliquots were analyzed by HPLC as the reaction progressed. A series of chromatographs is shown in figure 3. The primary product of the reaction is cGDPR, which had a retention time of 11.5 min and is well separated from NGD^+. A minor portion was hydrolyzed to GDP-ribose with a retention time of 15.9 min. The time courses of the reaction are shown in the inset. The initial rate of production of cGDPR was about 4 times higher than that of GDP-ribose. This is in striking contrast to the results obtained using NAD^+ as substrate as shown in Fig. 1 and 2. It has previous been shown that the accumulation of cGDPR during the reaction is due to its resistence to hydrolysis (18).

Another useful property of cGDPR is that it is fluorescent. Since neither NGD^+ nor GDP-ribose are fluorescent, the reaction can easily be monitored fluorimetrically. As shown in figure 4, incubation of 40 μM NGD^+ with two different concentrations of membranes from RA treated cells resulted in a progressive increase in cGDPR fluorescence, which can be calibrated by comparison with cGDPR standards. The rate of cGDPR production was determined to be 2.38 ± 0.24 nmol/mg protein/min, n=3. There was no measurable synthesis of cGDPR catalyzed by control membranes.

These results clearly show that the CD38 induced by RA in HL-60 cells has both ADP-ribosyl cyclase and cADPR hydrolase activities just like other CD38 proteins that have been



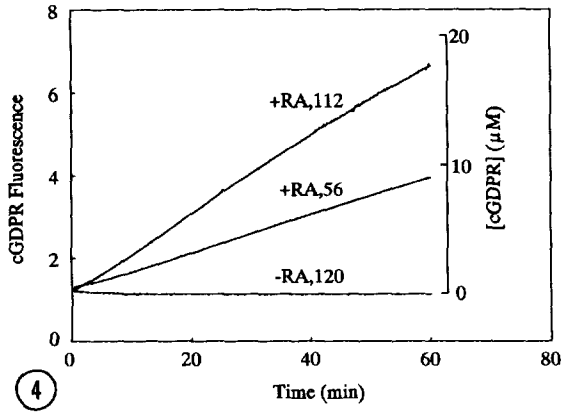


Figure 3. HPLC assay of GDP-ribosyl cyclase activity. Membrane from untreated (0.632 mg protein/ml) and RA treated (0.512 mg protein/ml) cells were incubated with 1 mM NGD^+ at 37°C and aliquots from the reactions were analyzed by HPLC. The numbers beside each chromatograph indicate the time of incubation. For the sake of clarity, the first 5 min of each chromatograph was not shown. The inset shows the time courses of substrate (NGD^+) and two products, cGDPR and GDP-ribose (GDPR). There was no detectable production of cGDPR in the untreated samples.

Figure 4. Fluorometric assay of GDP-ribosyl cyclase activity. Membranes (56 or 112 μg protein/ml) from RA-treated HL-60 cells ((+)RA56 and (+)RA112) were incubated with 40 μM NGD^+ for 1 hr and the fluorescence of cGDPR was monitored. The fluorescence intensity can be converted to cGDPR concentration by calibration with cGDPR standards. The control membranes (120 μg/ml) incubated similarly produced no increase in cGDPR fluorescence ((-)RA, 120).

described. The failure of previous studies in detecting cADPR production is due to the assays employed, which were not as sensitive as the Ca^{2+} release assay (12-14). Also shown in this study is that the cyclase activity of CD38 can be easily monitored by a novel assay using NGD^+ as substrate. The accumulation of cGDPR indicates that the cyclization reaction is a dominant reaction catalyzed by CD38. This fluorimetric assay eliminates the need for separating substrates and products by HPLC and is especially useful in identifying the presence of the CD38-like proteins in crude extracts. It is likely to be an invaluable tool for purifying this type of enzyme.

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