Expression of Cyclic ADP-Ribose–Synthetizing CD38 Molecule on Human Platelet Membrane

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CD38 is a cell surface molecule widely used as a marker for immature and activated lymphocytes. It has been recently shown that CD38 displays three enzymatic activities: hydrolysis of NAD⁺ to ADP-ribose, synthesis of cyclic ADP-ribose from NAD⁺, and hydrolysis of cyclic ADP-ribose to ADP-ribose. Thus, CD38 plays a key role in the synthesis of cyclic ADP-ribose, a calcium-mobilizing compound. We investigate here the expression and cellular localization of CD38 in human platelets using a specific monoclonal antibody. Results showed that CD38 is expressed by human platelet membranes. Moreover, we show that platelet CD38 possesses NAD glycohydrolase, ADP-ribose cyclase, and cyclic ADP-ribose hydrolase activities. This finding indicates that the calcium-mobilizing agent cyclic ADP-ribose can be synthesized by human platelets and raises the question about the possible role of CD38 expression and enzymatic activities in the signal transduction pathways leading to platelet activation.

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HUMAN CD38 IS A type II transmembrane glycoprotein expressed by discrete populations of human leukocytes, in which it appears to be involved in relevant cellular events such as activation, differentiation, and adhesion. Human, murine, and rat CD38 show great similarity both in amino acid sequence and function with the ADP ribosyl cyclase purified from Aplysia californica and from Aplysia kurodaya, an enzyme that converts β-NAD⁺ into cyclic ADP-ribose (cADPR). The catalytic properties shared by these molecules from such phylogenetically distant organisms were confirmed by recombinant human and murine CD38, which are able to catalyze the synthesis of cADPR from β-NAD⁺. CADPR has been reported to be a second messenger operating in different cell systems and promoting inositol 1,4,5-trisphosphate (IP₃)-independent Ca²⁺ mobilization from discrete intracellular stores. CD38 was recently found on the membrane of human erythrocytes, where it catalyzes the formation of cADPR from NAD⁺ and the hydrolysis to ADP-ribose (ADPR). The sum of these two activities results in the conversion of NAD⁺ to ADPR. Thus, CD38 appears to be a multicatalytic enzyme possessing NAD glycohydrolase (NADase), ADP-ribose cyclase, and cADPR hydrolyase activities.

Although CD38 is expressed by different blood and bone marrow cells, until now no evidence has been reported concerning its occurrence on platelet membranes. Platelets are very particular cell fragments that exhibit highly active signal transduction pathways and undergo, upon activation, profound structural and functional changes. This report shows that CD38 is present on the membrane of human platelets and that both intact cells and isolated membranes display ADP-ribose cyclase, cADPR hydrolase, and NADase activities. We also show that surface CD38 is responsible for all these enzymatic activities observed on platelets. Because platelets undergo profound structural and functional changes upon activation, they could serve as a model for studying the involvement of CD38 in the different signalling pathways which lead to cell activation. These results also raise stimulating questions on the role of CD38 in platelet functions.

MATERIALS AND METHODS

Materials. β-Nicotinamide adenine dinucleotide (β-NAD⁺), nicotinamide guanine dinucleotide (NGD⁺), ADP-ribose, ATP, ADP, AMP, ADP-ribose cyclase from Aplysia californica, bovine serum albumin (BSA), aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), Nonident P40, N-hydroxysuccinimidobiotin, fluorescein (FITC)-conjugated antimouse goat IgG were from Sigma (St Louis, MO). cADPR was a kind gift from A. De Flora (Institute of Biological Chemistry, University of Genoa, Genoa, Italy). The characteristics of the monoclonal antibody (MoAb) IB2 against CD38 are reported elsewhere. MoAb against platelet glycoprotein (GP) IIb-IIIa was from AMAC Immunotech (Westbrook, ME), and peroxidase-conjugated avidin was obtained from Dako (Glostrup, Denmark). Nitrocellulose membrane was purchased from Schleicher & Schuell (Dassel, Germany). Sepharose CL-2B and protein A-Sepharose were from Pharmacia Biotech (Uppsala, Sweden). Enhanced chemiluminescence (ECL) reagents and Hyperfilm ECL were from Amersham (Little Chalfont, UK). The bichromonic acid (BCA) protein determination kit was from Pierce (Rockford, IL). All other reagents were of analytical grade.

Platelet preparation. Blood was taken from healthy volunteers from the antecubital vein using citric acid-citrate-dextrose as anticoagulant. Platelets were isolated by gel filtration on a Sepharose CL-2B column equilibrated with HEPES buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 2.9 mmol/L KCl, 12 mmol/L NaHCO₃, pH 7.4), as described. The platelet count was finally adjusted to 10⁷ cells/mL.

Flow cytometry. Blood obtained from normal volunteers was anticoagulated with 10 mmol/L EDTA. Platelet-rich plasma was obtained by centrifuging the whole blood at 120g for 10 minutes at room temperature and treated as reported. Briefly, platelets were fixed in plasma at room temperature for 5 minutes with 2% (wt/vol) paraformaldehyde, centrifuged at 400g for 10 minutes, and resuspended in phosphate-buffered saline (PBS; 10 mmol/L NaH₂PO₄, 140 mmol/L NaCl, pH 7.4), containing 3 mmol/L EDTA.

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Reactive bands were evidentiated by a chemiluminescent reaction and 5% (wt/vol) BSA. Cells were then centrifuged at 400g for 10 minutes and resuspended in PBS, 0.3 mmol/L EDTA, 5% BSA, pH 7.4. Platelet samples were then incubated in the dark at room temperature for 30 minutes of incubation in the dark at room temperature. Cells were then washed and resuspended in 500 to 600 µL of filtered PBS, 0.3 mmol/L EDTA, 0.1% BSA, pH 7.4. Samples were analyzed by FACStar flow cytometer (Becton Dickinson, Mountain View, CA) with argon laser excitation.

Biocytinization of intact platelets. Gel-filtered platelets were incubated with 100 µg/mL of N-hydroxysuccinimidobiotin for 1 hour at room temperature. Cells were then biocytinized again on the same column used for platelet preparation. N-hydroxysuccinimidobiotin was dissolved at 10 mg/mL in dimethylsulfoxide (DMSO); the final concentration of DMSO in platelet samples did not exceed 1% (vol/vol).

Membrane preparation. Platelet concentrates were obtained from the local blood bank (Servizio di Immunematologia e Trasfusioni, IRCCS Policlinico S. Matteo, Pavia, Italy). Platelets were centrifuged at 1,800g for 20 minutes at room temperature and resuspended in a buffer containing 135 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L NaHCO₃, 0.36 mmol/L Na₂HPO₄, 2 mmol/L MgCl₂, 0.2 mmol/L EGTA, 5.5 mmol/L glucose, 0.35% (wt/vol) BSA, pH 6.3. Cells were then washed in the same buffer without EGTA, glucose, or BSA and finally resuspended at a final count of 2 x 10⁹ cells/mL in hypotonic buffer (10 mmol/L HEPES, 100 µg/mL PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, pH 7.5). Platelets were cooled at 4°C and disrupted with a Labsonic 2000 sonicator (Terzano, Milano, Italy) at maximal output. Sonicate was centrifuged at 1,800g for 20 minutes. Cleared lysate (500 µL) was incubated with 100 µg/mL IB4 MoAb or control MoAbs for 2 hours at 4°C. Samples were then added with 20 µg/mL leupeptin, 10 µg/mL aprotinin, pH 7.5. Cells and then ultracentrifuged at 100,000g at 4°C for 45 minutes. Cytosol was collected and frozen, and membranes were washed once with hypotonic buffer. The membrane protein concentration was determined by BCA assay and adjusted to 3 to 7 mg/mL. Membranes were stored at -70°C until used.

Purity of platelet preparations. Platelet preparations were checked for contamination by other cells both by direct microscope evaluation of May-Grünwald-Giemsa-stained smears of the samples and by cytofluorimetric determination of erythrocytes and B lymphocytes. Both approaches showed that the contamination of platelet samples by other cells was absent or negligible (data not shown).

Immunoprecipitation. Platelets or membranes were lysed by adding 1 vol of ice-cold immunoprecipitation buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 2.9 mmol/L KCl, 12 mmol/L NaHCO₃, 2% [vol/vol] Nonidet P40, 1% [vol/vol] sodium deoxycholate, 0.2% [wt/vol] sodium dodecyl sulfate [SDS], 1 mmol/L PMSF, 20 µg/mL aprotinin, 20 µg/mL leupeptin, pH 7.5). Lysates were kept in ice for 15 minutes and then centrifuged at 13,000g for 5 minutes. Cleared lysate (500 µL) was incubated with 8 µg/mL IB4 MoAb or control MoAbs for 2 hours at 4°C. Samples were then added with 100 µL of protein A-Sepharose dissolved in deionized water at 50 mg/mL and incubated for 30 minutes at 4°C. The protein A-Sepharose pellets were collected by brief centrifugation and washed five times with ice-cold immunoprecipitation buffer diluted 1:1 with HEPES buffer. Immunoprecipitates were then directly dissociated with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, loaded on 10% PAGE gels, electrophoretically transferred to nitrocellulose, and then probed with avidin-peroxidase. Reactive bands were evidenced by a chemiluminescent reaction (Amersham). Immunoprecipitates were resuspended in 10 mmol/L HEPES, pH 7.5, for enzymatic analysis.

Enzymatic analysis. For enzymatic analysis, intact platelets were suspended in HEPES buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 2.9 mmol/L NaHCO₃, 12 mmol/L NaHCO₃, pH 7.4) and platelet membranes and immunoprecipitates were suspended in 10 mmol/L HEPES, pH 7.5. Samples were prewarmed at 37°C for 5 minutes and then rendered 1 mmol/L NAD⁺ for NADase and ADP-ribosyl cyclase assays, 100 µmol/L cADPR for cADPR hydrolase assay, and 100 µmol/L NGD⁺ for guanosine 5'-diphosphoribosyl cyclase (GDP-ribosyl cyclase) assay. Aliquots (100 µL) were taken at different times of incubations (ranging from 0 to 60 minutes) at 37°C and centrifuged at 13,000g for 5 minutes. Supernatants were collected and ultrafiltered on Amicon Microcon-3 membrane (molecular weight cut off, 3,000 Daltons). Filtered samples were then analyzed by reverse-phase high pressure liquid chromatography (HPLC).

HPLC analysis. The reactions of ADP-ribosyl cyclase, cADPR hydrolase, NAD glycohydrolase, and GDP-ribosyl cyclase activities of intact platelets, membranes, and immunoprecipitates were monitored by HPLC and quantified with calibration curves of standard NAD⁺, NGD⁺, cADPR, and ADP. Cyclic guanosine diphosphate-ribose (cGDP) was obtained by incubating known amounts of NGD⁺ with Aplysia ADP-ribosyl cyclase, as described. The nucleotides were separated on a 25 x 0.46 cm Supelcosil LC-18 reversed phase column (Supelco, Bellefonte, PA). The flow rate was maintained at 1.3 mL/min and nucleotides were eluted with a gradient from 0% to 10% methanol, obtained by mixing buffer A (0.1 mol/L KH₂PO₄, pH 6.00) and buffer B (0.1 mol/L KH₂PO₄, 10% vol/vol methanol, pH 6.00), as described. Briefly, buffer B was held to 0% for 9 minutes, increased linearly to 25% by 15 minutes, increased linearly to 90% from 15 to 17.5 minutes, stepped to 100% by 19.5 minutes, and held at 100% until 25.5 minutes. Nucleotides of interest were eluted before 22 minutes. The column was calibrated with β-NAD⁺, cAMP, ADP, ATP, cADPR, ADPR, NGD⁺, and cGDP. The peaks were detected at 254 nm.

RESULTS

Cytofluorimetric analysis. A significant fluorescence signal was obtained after reacting platelets with IB4, an MoAb specific for human CD38, whereas negligible fluorescence was present on platelet incubated with unrelated antigens (anti-HLA II) or with the second antibody alone (Fig 1). Also, the positive control performed using an anti-GBP1b-IIIa MoAb gave, as expected, a strong fluorescence signal (data not shown).

Immunoprecipitation of biotinylated intact platelets. Platelets were biotinylated with 100 µg/mL of N-hydroxysuccinimidobiotin for 1 hour at room temperature and then lysed with immunoprecipitation buffer as described in the Materials and Methods. Immunoprecipitation was performed by adding the IB4 MoAb or isotype-matched control MoAbs directed against surface molecules, eg, HLA class II, not expressed by the platelets. Immunoprecipitates were then subjected to SDS-PAGE, Western blotting, and avidin-peroxidase staining. IB4-immunoprecipitates from intact biotinylated resting platelets showed a single band with a molecular mass of 46 kD (Fig 2, lane 2). This band had the same electrophoretic mobility of that observed in the IB4-immunoprecipitate from a CD38⁺ B-lymphoma cell line (data not shown). The band was not
preparations incubated with NGD' however, we were not able to detect cGDPR production (data not shown). This indicates that cyclase activity is located exclusively on platelet membranes. To further investigate the cellular topology of the enzyme activities, intact platelets were used to perform the determinations. Using NAD' as the substrate, both NADase and hydrolase activities could be detected (data not shown). Moreover, platelet incubation with NGD' resulted in the production of cGDPR in a manner similar to that shown in Fig 4 (data not shown), thus confirming that NADase, ADP-ribosyl cyclase, and cADPR hydrolase are detectable in intact platelets and are therefore exposed at the outer surface of the membrane.

Platelet CD38 accounts for NAD glycohydrolase, ADP-ribosyl cyclase and cADPR hydrolase activities. To test whether the CD38 protein was responsible for the formation and hydrolysis of cADPR, platelet membranes were solubilized with immunoprecipitation buffer and incubated with the IB MoAb. The immunoprecipitate was resuspended in 100 μL of 10 mmol/L HEPES, pH 7.5,

Enzyme activities determination. Plasma membrane preparations were obtained as described from platelet concentrates and were used to assay NADase, ADP-ribosyl cyclase, and cADPR hydrolase activities. When platelet membranes were incubated with 1 mmol/L NAD' at 37°C, a peak corresponding to ADPR and progressively increasing with time appeared in the HPLC chromatogram (Fig 3A). A much less evident peak corresponding to cADPR could also be detected (Fig 3A).

The cADPR hydrolase activity of membrane preparations was measured by incubating samples with 100 μmol/L cADPR at 37°C for increasing times; in these conditions, production of ADPR was clearly detected by HPLC analysis (Fig 3B). When the substrates or the membranes were not present in the incubation mixture, the formation of the products was completely absent. The specific activities expressed as nanomoles of products formed per minute by 1 mg of membrane proteins (Table 1) indicate a NADase/cADPR hydrolase/ADP-ribosyl cyclase ratio of about 100:6:1. In our experimental conditions, the production of cADPR was clearly detectable, even if small; cADPR was a substrate for the cADPR hydrolase activity. For this reason, the presence of the ADP-ribosyl cyclase activity was also studied using NGD' instead of NAD' as substrate; this compound is converted by CD38 to cGDPR, which, in contrast to cADPR, is a poor substrate for the hydrolase activity. This allows accumulation of the cyclic product that can be easily detected by HPLC. Platelet membranes in the presence of 100 μmol/L NGD' produce cGDPR, as shown by HPLC analysis (Fig 4). Cyclase activity assays were also performed with platelet cytosol present when the negative control MoAb (HLA class II) was used (Fig 2, lane 1).

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Fig 3. NADase, ADP-ribosyl cyclase, and cyclic ADPR hydrolase activities in platelet membranes. Platelet membranes (100 µL, 6 to 7 mg/mL of proteins) resuspended in 10 mmol/L HEPES, pH 7.5, were incubated for increasing times at 37°C with 1 mmol/L B-NAD⁺ (A) or 100 µmol/L cADPR (B), centrifuged, and ultrafiltered on Amicon Microcon-3 membrane. Twenty-microliter aliquots were then subjected to HPLC analysis. Cyclic ADP-ribose and ADP-ribose were quantified by using a calibration curve obtained with known amounts of the standard nucleotides. The plots show the amounts of cADPR and ADPR produced at different times by 1 mg of membrane proteins. Data of a typical experiment of 6 different preparations that gave similar results are reported.

DISCUSSION

The purpose of this study was to investigate the presence of the CD38 molecule on platelet membranes and to examine its catalytic features, i.e., its reported ADP-ribosyl cyclase and cADPR hydrolase activity in several cell types.

Using the murine MoAb IB₄ specific for human CD38, we showed using cytofluorimetric analysis that the molecule is significantly expressed by platelets. Confirmation of this observation was obtained by immunoprecipitation experiments from biotinylated platelets using the same IB₄ MoAb; avidin-peroxidase staining evidenced the expected single band of 46 kD after SDS-PAGE and Western blotting. Because biotin in these experiments can react only with proteins exposed to the extracellular side of the membrane, this result highlights the surface localization of CD38. Plasma membrane preparations were able to convert NAD⁺ to ADPR; additionally, in the same incubation mixture, the formation of cADPR was detected by reverse-phase HPLC. Because the levels of this compound were very low, the cyclase activity was also assayed using NGD⁺ as the substrate. NGD⁺ is converted by the same cyclase to cGDPR, which can only be poorly hydrolyzed to GDPR. In these experimental conditions, a significant cyclase activity was detectable in the membrane preparations. The cADPR hydrolase activity is present in the membrane preparation, because the formation of ADPR is stoichiometric with the disappearance of cADPR. All the catalytic activities under investigation were expressed on the extracellular side of the membrane, demonstrated also by the fact that intact platelets were able to use NAD⁺, cADPR, and NGD⁺. Moreover, the same enzymatic activities can be recovered in the IB₄ MoAb immunoprecipitates, confirming that the CD38 molecule is a multifunctional enzyme also in platelets. It remains unclear whether the production of cADPR is an obligatory step in the conversion of NAD⁺ to ADPR or whether a direct NADase activity is present together with ADP-ribosyl cyclase and cADPR hydrolase. However, the evidence that the ratio NADase/cADPR hydrolase/ADP-ribosyl cyclase is similar in mem-

and enzyme activities were tested (Fig 5). Despite the presence of the IB₄ MoAb, the enzyme activities could be detected in the samples, confirming previous observations concerning the independence of the CD38 catalytic domain from the IB₄ epitope. Also, in this case, NAD⁺ could be transformed by the cyclase to the corresponding cyclic product (Fig 5A). Moreover, the IB₄ immunoprecipitate clearly produced ADPR when NAD⁺ or cADPR were used as the substrate (Fig 5A and B). The ratio between NADase/cADPR hydrolase/ADP-ribosyl cyclase is similar to that observed using platelet membrane preparations, thus confirming that CD38 is responsible for all the enzymatic activities under investigation.

Table 1. NADase, ADP-Ribosyl Cyclase, and cADPR Hydrolase Specific Activities of Platelet Membranes

<table>
<thead>
<tr>
<th>Activity</th>
<th>Specific Activities (mU/mg of protein)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADase</td>
<td>2.16</td>
<td>100</td>
</tr>
<tr>
<td>cADPR hydrolase</td>
<td>0.132</td>
<td>6</td>
</tr>
<tr>
<td>ADP-ribosyl cyclase</td>
<td>0.024</td>
<td>1</td>
</tr>
</tbody>
</table>

One unit of enzyme activity is defined as the amount of enzyme that produces 1 µmol of ADPR (NADase) or cADPR (ADP-ribosyl cyclase) from NAD⁺ and 1 µmol ADPR (cADPR hydrolase) from cADPR per minute at 37°C, under the conditions described in the Materials and Methods.
brane preparations to that in IB4 MoAb immunoprecipitates strongly suggests that, in any case, CD38 is responsible for all the enzymatic activities converting NAD to ADPR. The finding that CD38 is present on platelet membrane raises the intriguing question of its function. At present it is only possible to speculate, but it is interesting that platelets undergo many of the processes in which CD38 and its enzymatic products have already been reported to be involved in several cell types. Platelets exhibit many signal transduction pathways, including Ca²⁺ movement, are regulated in their function by different mechanisms, and adhere to each other and to other cell types. These cells therefore represent a very stimulating model for future research on the biologic role of CD38 and may provide valuable information for the identification of its receptor.

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Fig 4. Platelet membranes produce cGDPR from NGD⁺. Typical chromatographic pattern obtained after incubation for 30 seconds (A) or for 30 minutes (C) of membrane samples at 37°C with 100 µmol/L NGD⁺. The peaks corresponding to NGD⁺ and cGDPR are shown. cGDPR was identified by coelution with standard cGDPR obtained by incubation of 100 µmol/L NGD⁺ with 3 µg of Aplysia californica ADP-ribosyl cyclase for 60 minutes at room temperature (B). The peak marked by an asterisk is the putative GDPR.²¹

Fig 5. NADase, ADP-ribosyl cyclase, and cADPR hydrolase activities in IB4-immunoprecipitates. The immunoprecipitates obtained from 250 µL of membranes (6 to 7 mg/mL) and resuspended in 10 mmol/L HEPES, pH 7.5, were directly incubated with 1 mmol/L NAD⁺ (A) or 100 µmol/L cADPR (B) (final volume, 100 µL); ADPR and cADPR were quantified by HPLC analysis. The figure shows a typical time course of ADPR and cADPR production from NAD⁺ and of ADPR production from cADPR. The results are expressed as nanomoles of product obtained per milliliter of incubation suspension.
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