Stimulation of ADP-ribosyl cyclase activity of the cell surface antigen CD38 by zinc ions resulting from inhibition of its NAD⁺ glycohydrolase activity

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The lymphocyte cell surface antigen, CD38, which has an amino acid sequence similar to *Aplysia* ADP-ribosyl cyclase, catalyzes not only the hydrolysis of NAD⁺ and 1-(5-phospho-β-d-ribozy)adenosine 5'-phosphate cyclic anhydride (cyclic ADP-ribose) but also the formation of cyclic ADP-ribose from NAD⁺. To characterize the bifunctional enzyme properties, we produced the recombinant CD38 fused with a maltose-binding protein (MBP-CD38). Zinc ions stimulated the ADP-ribosyl cyclase activity of MBP-CD38, but inversely inhibited its NAD⁺ glycohydrolase activity which was approximately 100-fold dominant to the cyclase activity in the absence of Zn²⁺. Such dual effects of Zn²⁺ were also observed in the native membrane-bound CD38 of HL-60 cells which had been caused to differentiate by retinoic acid. Zinc ions inhibited the NAD⁺ glycohydrolase reaction catalyzed by MBP-CD38 in an uncompetitive manner, whereas they enhanced the ADP-ribosyl cyclase reaction without affecting the *Kₘ* value for NAD⁺. There was an increase in the fluorescence intensity of a hydrophobic fluorescent probe, 8-anilino-1-naphthalenesulfonate, in the presence of MBP-CD38. The fluorescence increase was further enhanced by the addition of Zn²⁺ with a shift in the maximum emission wavelength from 484 nm to 470 nm, suggesting that Zn²⁺ caused conformational changes of MBP-CD38. These results indicate that Zn²⁺ directly interacts with CD38 to stimulate its ADP-ribosyl cyclase with inhibition of its NAD⁺ glycohydrolase, probably due to prevention of the access of water molecule to an intermediate of the enzyme-substrate complex.

**Keywords:** CD38; cyclic ADP-ribose; zinc; NAD⁺ glycohydrolase; ADP-ribosyl cyclase.

The cell-surface antigen, CD38, is a 46-kDa type-II single-transmembrane glycoprotein with a short N-terminal cytoplasmic domain and a long C-terminal extracellular domain [1, 2]. The expression of CD38 is widely used as a phenotypic marker of the differentiation or activation of T and B lymphocytes [3–5], though its function has not been fully elucidated. We previously reported that an ecto-enzyme activity of NAD⁺ glycohydrolase induced by retinoic acid in HL-60 cells has been caused to differentiate by retinoic acid. ADP-ribosyl cyclase [6]. Cyclic ADP-ribose, which catalyzes the formation of 1-(5-phospho-β-d-ribozy)adenosine 5'-phosphate cyclic anhydride (cyclic ADP-ribose) from NAD⁺ [8–10]; there are especially 10 cysteine residues conserved between CD38 and *Aplysia* ADP-ribosyl cyclase [11]. Cyclic ADP-ribose has been considered as a new mediator of Ca²⁺ release from intracellular stores [12, 13].

It has been reported that CD38 catalyzes not only the hydrolysis of NAD⁺ but also the formation and hydrolysis of cyclic ADP-ribose [14–16]. However, the ADP-ribosyl cyclase activity of CD38 is quite low compared to its NAD⁺ glycohydrolase activity; the ratio of the two specific activities is approximately 1:100 [17]. In this respect, the ADP-ribosyl cyclase activity of CD38 in human erythrocyte membrane appeared to be stimulated by the addition of Cu²⁺ or Zn²⁺ [16], though its stimulatory mechanism has not been investigated yet. On the other hand, human BST-1, a surface molecule of bone marrow stromal cell lines that facilitates pre-B cell growth, has recently been reported to have an amino acid sequence similar to CD38 [18]. BST-1 protein expressed in Chinese hamster ovary cells displayed the enzyme activities of ADP-ribosyl cyclase and cyclic ADP-ribose hydrolyase in the presence of metal ions such as Zn²⁺ and Mn²⁺ [19]. In the present study, we produced the recombinant CD38 fused with a maltose-binding protein (MBP-CD38) in *Escherichia coli* and investigated the effects of various metal ions on its enzyme properties, together with that of the native membrane-bound CD38 in HL-60 cells. The ADP-ribosyl cyclase activity of CD38 was stimulated by Zn²⁺ with inhibition of its NAD⁺ glycohydrolase activity, and the metal ions induced conformational changes of CD38, which was measured by a fluorescent-dye probe.

**MATERIALS AND METHODS**

Construction and purification of MBP-CD38 fusion protein. The extracellular domain of human CD38 cDNA covering the 202–972-bp nucleotide positions was obtained by reverse-
transcriptase PCR with mRNA of HL-60 cells that had been subcloned into the Smal site of pBluescript II SK (−). To ensure that misincorporation of bases had not been generated by PCR, the amplified cDNA of human CD38 was completely sequenced by the dyeideoxy sequencing. Plasmid pMAL-cR1/Ext CD38, which encodes a fusion of MBP to the extracellular domain of CD38, was constructed as follows. A 0.8-kb Smal fragment of CD38 cDNA was cloned-in-frame into pMAL-cR1 (New England Biolabs) that had been digested with EcoRI and blunt-ended with T4 DNA polymerase. pMAL-cR1/Ext CD38 thus obtained was introduced into E. coli strain JM109 to produce MBP-CD38 fusion protein.

The bacterial cells were cultured at 37°C with vigorous shaking in 11 of an enriched medium (pH 7.0) consisting of tryptone (1.6%), yeast extract (1%) and NaCl (0.5%). After the culture reached an absorbance at 660 nm of 0.7–1.0 isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM. After an additional 1-h culture, the cells were harvested by centrifugation at 5000 × g for 5 min. The cells were resuspended in a lysis buffer consisting of 10 mM sodium phosphate pH 7.2, 30 mM NaCl, 0.25% Tween 20, 1 mM EDTA and 100 μM phenylmethylsulfonyl fluoride and frozen in a solid CO2/ethanol bath to facilitate cell lysis. The suspension was thawed in cold water and sonicated on ice for 2 min. After addition of NaCl to the final concentration of 0.5 M, the cell lysate was centrifuged at 9000 × g for 30 min and the clear supernatant was collected. The supernatant was applied to an amylose-affinity column (0.7 cm × 3 cm; New England Biolabs) which had been equilibrated with a buffer consisting of 10 mM sodium phosphate pH 7.2, 0.5 M NaCl, 0.25% Tween 20 and 1 mM EDTA, then the column was washed with 5 vol. equilibration buffer. MBP-CD38 was eluted with the same buffer containing 10 mM maltose. Coomassie brilliant blue staining of the eluted fraction exhibited a single band of MBP-CD38 (M, of 70 000) on SDS/PAGE.

MBP-CD38 eluted from the affinity column appeared to be slightly turbid; the majority of the fusion protein was eluted in a voided fraction upon its application to a Superose 12 gel filtration column, suggesting that it was aggregated. Therefore, MBP-CD38 eluted from the affinity column was treated with 6 M guanidine hydrochloride and then refolded by dialysis for 8 h twice against 100 vol. 20 mM Tris/HCl pH 8.0 and 0.7% Chaps. The refolded MBP-CD38 (20 ml) was concentrated to approximately 200 pg/ml and stored at 4°C until use. MBP alone was also produced at E. coli and purified by amylose-affinity chromatography as described above.

Preparation of HL-60 cell membrane. HL-60 cells were cultured and caused to differentiate by retinoic acid; crude cell membrane was prepared therefrom as described previously [6]. The majority of NAD+ -degrading activity in the membrane was due to CD38 induced by retinoic acid [6].

Assay of enzyme activities. For the determination of NAD+ glycohydrolase activity, MBP-CD38 (10 μg protein/ml) was incubated at 30°C for 10 min with 200 μM [α-32P]NAD+ (specific activity of 5 Ci/mol) in 20 μl 20 mM Tris/HCl pH 8.0, 100 mM NaCl and 0.7% Chaps containing various concentrations of divalent cations. After the incubation, a 4-μl aliquot was withdrawn and mixed with 2 μl 10% SDS. 1 μl of the sample was spotted on a poly(ethyleneimine)-cellulose sheet (Schleicher & Schüll) and developed in 0.2 M NH4HCO3, (R, values of NAD+ and ADP-ribose were 0.51 and 0.11, respectively). The radioactivity of [32P]ADP-ribose was determined with a Fuji BAS 2000 imaging analyzer.

For the determination of ADP-ribosyl cyclase activity, MBP-CD38 (10 μg protein/ml) was incubated with 200 μM [α-32P]NAD+ under the same conditions as described above. The reactions were stopped by the addition of trichloroacetic acid to a final concentrations of 20%. After the extraction of trichloroacetic acid with ethyl ether, 4 μl of the sample was mixed with 2 μl snake venom phosphodiesterase (3000 units/ml; Worthington Biochemical Co.) in 250 μM Tris/HCl pH 9.0 and 25 mM MgCl2, and further incubated for 5 min at 37°C. This enzyme treatment converts the substrate, [α-32P]NAD+, into [α-32P]AMP, without degradation of the product, cyclic [32P]ADP-ribose [20]. 1 μl of the enzyme-treated sample was then subjected to the thin-layer chromatography as described above (R, values of cyclic ADP-ribose and AMP were 0.53 and 0.28, respectively). The concentration of cyclic ADP-ribose formed in the reaction with the enzyme was also determined by a radioimmunoassay specific for cyclic ADP-ribose as described previously [21].

Fluorescence measurement. The fluorescence of 8-anilino-1-naphthalenesulfonate (purchased from Wako Pure Chemical, Osaka) was measured using a F-2000 spectrophotometer (Hitachi) in a water-jacketed cuvette held at 25°C. To measure its fluorescence spectrum it was added to a final concentration of 20 μM to cuvettes containing MBP-CD38 (20 μg protein/ml) in 20 mM Tris/HCl pH 8.0 and 100 mM NaCl. The solution also contained various metal ions. The fluorescence intensity was monitored between 420–620 nm with an excitation wavelength of 360 nm.

RESULTS

Zinc-induced changes of the enzyme properties of CD38. The fusion protein of MBP-CD38 was incubated with NAD+ in the presence of various metal ions (1 mM), and enzyme activities of NAD+ glycohydrolase and ADP-ribose cyclase were then measured (Fig. 1). Zn2+, Cd2+ and Ca2+ stimulated ADP-ribose cyclase activity of MBP-CD38, but inversely inhibited NAD+ glycohydrolase activity. Similar effects were observed with Ni2+ and Co2+ (data not shown). Among these metal ions, Zn2+ was most effective with regard to both inhibition of NAD+ glycohydrolase and stimulation of ADP-ribose cyclase. On the other hand, Mn2+, Ca2+ and Mg2+ had no influence on the enzyme activities of MBP-CD38. Ba2+ and Sr2+ also had no effect on the enzyme activities (data not shown).

The effects of very low concentrations of Zn2+ on the two enzyme activities were next investigated, and the results are illustrated in Fig. 2. The NAD+ glycohydrolase activity was progressively inhibited as the concentration of Zn2+ was increased. On the contrary, there was an increase in the ADP-ribose cyclase activity under the same conditions that the cation concentration was increased. The cyclase activity observed in the pres-
The data are expressed as percentages of the control values in the absence of metal ions, which were $523 \pm 18$ and $2.5 \pm 0.2 \text{ mmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of NAD$^+$ glycohydrolase and ADP-ribosyl cyclase activities, respectively. Results are the mean $\pm$ SE of three independent experiments.

Kinetic analysis of the effect of Zn$^{2+}$ on the two enzyme activities of CD38. In an attempt to determine the mode of the inhibitory action of Zn$^{2+}$ on the NAD$^+$ glycohydrolase activity of CD38, initial velocities of ADP-ribose production by MBP-CD38 were measured versus various concentrations of NAD$^+$. (A) MBP-CD38 (100 pmol) was incubated at 30°C for 10 min with various concentrations (0.01–0.2 mM) of [$\alpha$-$^3$P]NAD$^+$ in the presence of 0.1 mM (●), 0.3 mM (▲), or 1 mM (●) ZnCl$_2$ or in its absence (○). [$^3$P]ADP-ribose produced was measured as described in Materials and Methods. The data are representative of three independent experiments. (B) The double-reciprocal plots of the data in A.

Zinc-induced fluorescence enhancement of 8-anilino-1-naphthalenesulfonate bound to MBP-CD38. 8-Anilino-1-naphthalenesulfonate is an anionic amphiphile used as a probe for hydrophobic regions of proteins [22]. As shown in Fig. 6, there was a marked enhancement of its fluorescence in the presence of MBP-CD38; this enhancement was accompanied with a shift in the maximum emission wavelength from 520 nm to 484 nm. Such a large enhancement was not observed, however, in MBP-CD38.
ADP-ribosyl cyclase activity (nmol/min/mg) was incubated at 30°C for 10 min with various concentrations of Zn2+ in the presence of 0.1 mM (●), 0.3 nm (△), or 1 mM (▲) ZnCl2, or in its absence (○). Cyclic [32P]ADP-ribose produced was measured as described in Materials and Methods. The data are representative of three independent experiments. (B) The double-reciprocal plots of the data in A.

**Fig. 5. Kinetics of ADP-ribosyl cyclase activity of MBP-CD38 in the presence of various concentrations of Zn2+.**

**Fig. 6. Fluorescence spectrum of 8-anilino-1-naphthalenesulfonate bound to MBP-CD38 as enhanced by Zn2+.** The fluorescent emission spectrum of 8-anilino-1-naphthalenesulfonate was monitored with the excitation wavelength of 360 nm in the presence of MBP-CD38 (20 μg protein/ml) under various conditions as described in Materials and Methods. (a) 8-Anilino-1-naphthalenesulfonate alone (minus MBP-CD38); (b) MBP-CD38; (c) MBP-CD38 plus 1 mM ZnCl2; (d) c plus 2 mM EDTA; (e) MBP-CD38 denatured by 6 M urea; (f) MBP (12 μg protein/ml); (g) MBP plus 1 mM ZnCl2; (h) MBP-CD38 plus 1 mM CaCl2; (i), MBP-CD38 plus 1 mM MgCl2. The inset shows the concentration-dependent effect of Zn2+ on the fluorescence intensity of 8-anilino-1-naphthalenesulfonate at the emission wavelength of 470 nm.

CD38 which had been denatured by 6 M urea so the enhancement appeared to be due to its interaction with hydrophobic regions of the intact MBP-CD38.

**Fig. 7. Proposed mechanisms for NAD+ glycohydrolase and ADP-ribosyl cyclase reactions catalyzed by CD38.** E, NA, cADPR, ADPR, and [E·ADPR+] represent CD38, nicotinamide, cyclic ADP-ribose, ADP-ribose, and an intermediate of the enzyme-ADP-ribose oxocarbonium ion, respectively. The arrow with minus symbol indicates a site for the Zn2+-induced inhibition.

**DISCUSSION**

Ten cysteine residues are highly conserved among human [2], mouse [23], and rat [24] CD38s, human [18] and mouse [25] BST-1s, and _Apysia_ ADP-ribo-sy cyclase [10]. In the previous study, we reported that the NAD+ glycohydrolase activity of CD38 in HL-60 cells is remarkably inhibited by a reducing agent of dithiothreitol [6]. This suggests that a disulfide bond(s) and/or a free Cys are responsible for conservation of the tertiary structure to express the enzyme activity. In this study, we have found that the NAD+ glycohydrolase activity of CD38 is markedly abolished, whereas its ADP-ribo-sy cyclase activity is stimulated, in the presence of Zn2+; the dual action of Zn2+ is reversibly observed. These results indicate that Zn2+ directly binds to CD38, leading to the changes of its catalytic properties.

As has been discussed in the NAD+ glycohydrolase of canine spleen [20], an enzyme-stabilized intermediate form of ADP-ribo-sy oxocarbonium ion would be postulated in the NAD+ glycohydrolase reaction catalyzed by CD38 (Fig. 7). This intermediate could react with water to yield ADP-ribo-sy rapidly and sequentially. Similar to the spleen NAD+ glycohydrolase, CD38 also produced NAD+ from cyclic ADP-ribose and nicotinamide in a considerable extent [17]. This suggests that cyclic ADP-ribose can interact with CD38 to form their complex, which might be equivalent to the intermediate generated from the enzyme-NAD+ complex after nicotinamide release. It is, therefore, very likely that the hydrolase and cyclase reactions catalyzed by CD38 share the common intermediate of the enzyme-ADP-ribo-sy oxocarbonium ion. Only a small fraction of this intermediate can be converted to cyclic ADP-ribo-sy under usual conditions, because of its rapid conversion to ADP-ribo-sy by the attack of water molecule. Zinc ions inhibited the NAD+ glycohydrolase reaction catalyzed by MBP-CD38 in an uncompetitive manner (see Fig. 4), whereas they enhanced the ADP-ribo-sy cyclase reaction without affecting the Kcat value for NAD+ (see Fig. 5). The uncompetitive inhibition with regard to NAD+ indicates that Zn2+ interacts with an enzyme-substrate complex and exerts its inhibitory effect on the NAD+ glycohydrolase reaction (see Fig. 7). Moreover, Zn2+ enhanced the fluorescence intensity of 8-anilino-1-naphthalenesulfonate bound to MBP-CD38, suggesting that Zn2+ caused the exposure of a domain of CD38 with considerable hydrophobic character (see
Fig. 6). The concentration-dependent effect of Zn\(^{2+}\) on the fluorescence change was not exactly the same as that on the enzyme activities (see Figs 2 and 6). This discrepancy may suggest that only small conformational changes are adequate to induce the changes of the enzyme properties of CD38. In conclusion, Zn\(^{2+}\) could cause the conformational changes of CD38 and prevent the access of water molecule to the enzyme-ADP-ribosyl oxocarbonium ion, resulting in the elevated production of cyclic ADP-ribose.

Although CD38 dose not contain typical zinc-binding motifs, such as a zinc finger, zinc cluster or zinc twist, which are included in DNA-binding zinc proteins [26], there are ten conserved cysteine residues which could serve as ligands to zinc ions. Recently, it has been reported that Cys119 and Cys201 in human CD38 play a crucial role in the synthesis and hydrolysis of cyclic ADP-ribose [27]. Mutated CD38, in which Cys119 and/or Cys201 had been substituted with other amino acids, displayed only ADP-ribosyl cyclase activity [27]. It is thus likely that Zn\(^{2+}\) binds CD38 through Cys119 and Cys201 which are conserved among all CD38 but not in Aplysia ADP-ribosyl cyclase. In other words, Cys119 and/or Cys201 would be responsible, at least in part, for the acceptance of water molecule in the enzyme-substrate complex, leading to the hydrolyase-type enzyme. Thus, the direct binding of Zn\(^{2+}\) to the Cys residue(s) would result in inhibition of NAD\(^{+}\) glycohydrolase and stimulation of ADP-ribosyl cyclase. CD38 has an amino acid sequence similar to BST-1 [18, 25]. However, there is a difference in sensitivity to Mn\(^{2+}\) between CD38 and BST-1 [25]. ADP-ribosyl cyclase activity of BST-1 was stimulated by the metal ion but that of CD38 was not. This may be due to a different selectivity for ionic radius in the putative metal-binding site between CD38 and BST-1.

It has been reported that a low level (approximately 100 µg/100 ml) of Zn\(^{2+}\) exists, mostly being bound to albumin loosely, in human plasma [28]. This implies that CD38 on the surface of human hematopoietic cells binds Zn\(^{2+}\) in the physiological conditions and exerts its ADP-ribosyl cyclase activity as an effective enzyme profile. Cyclic ADP-ribose applied externally can potentiate B lymphocyte proliferation in the presence of infectious enzyme profile. Cyclic ADP-ribose applied externally for ionic radius in the putative metal-binding site between CD38 and/or Cys201 had been substituted with other amino acids, dissociated significant conformational changes and prevented the access of water molecule to the enzyme-ADP-ribosyl oxocarbonium ion, resulting in the elevated production of cyclic ADP-ribose.

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